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REVIEW

Cytomegalovirus in human brain tumors: Role in pathogenesis and potential treatment options

Cecilia Söderberg-Nauclér, John Inge Johnsen

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Abstract

During the last years increasing evidence implies that human cytomegalovirus (CMV) can be attributed to human malignancies arising from numerous tissues. In this perspective, we will review and discuss the potential mechanisms through which CMV infection may contribute to brain tumors by affecting tumor cell initiation, progression and metastasis formation. Recent evidence also suggests that anti-CMV treatment results in impaired tumor growth of CMV positive xenografts in animal models and potentially increased survival in CMV positive glioblastoma patients. Based on these observations and the high tumor promoting capacity of this virus, the classical and novel antiviral therapies against CMV should be revisited as they may represent a great promise for halting tumor progression and lower cancer deaths.

Key words: Cytomegalovirus; Oncovirus; Glioblastoma; Medulloblastoma; Brain tumor

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Core tip: Cytomegalovirus (CMV) has recently been detected in several human cancers. These findings have raised several concerns whether this virus is the cause or a passenger during oncogenesis. Here we discuss the pathogenesis behind CMV infection, its potential as an onco- or oncomodulatory-virus and possible modes of medical interventions.

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HUMAN CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is a common virus belonging to the herpesvirus family[1], which infects 70%-100% of the world's population. After a primary infection that is generally mild or asymptomatic in the immunocompetent host, this virus establishes latency and persistence in myeloid lineage cells, and periodic asymptomatic reactivations are believed to occur during life[1] without clinical signs of infection. However, in individuals with a suppressed immune system, CMV may cause life-threatening infections. Despite a good prophylaxis and surveillance program for early detection of reactivation of CMV in transplant patients, these infections remain to be a clinical problem both as an acute infection as well as a cause of long-term complications. Patients who have had CMV infections in the post-transplant period are at higher risk of developing acute and chronic rejection, cardiovascular diseases, bacterial and fungal infections, post-transplant diabetes and some malignancies[2-5]. These conditions are mainly believed to be mediated by indirect effects of CMV[4], as the virus has been difficult to detect in affected organs at time of diagnosis[6]. However, more recently, the use of sensitive techniques for detection of CMV demonstrate that the presence of CMV in kidney grafts is associated with decreased organ function and graft survival[7,8], suggesting that this virus causes direct, in addition to indirect effects, in the graft.

Emerging evidences also suggest that CMV is highly prevalent in patients with breast, colon, and prostate cancer, rhabdomyosarcoma, hepatocellular cancer, salivary gland tumors, neuroblastoma and brain tumors[9-25]. Over 90% of these tumors have been shown to be positive for CMV proteins and nucleic acids as determined by methods including in situ hybridisation, PCR, electron microscopy, DNA and RNA sequencing, immunostaining of tissue specimens, flow cytometry analyses of tumor cells from surgical resections and western blot analysis. Also most neoplastic cells in sentinel lymph nodes of > 90% of breast cancers have been shown to be CMV positive[26] as well as 98% of brain metastases of colon and breast cancers contain CMV proteins and/or nucleic acids[27]. In sharp contrast, CMV proteins are not detected in healthy tissues surrounding CMV positive tumors or metastases. These observations suggest that this virus does not represent an epiphenomenon of CMV positive tumors, but rather that it may directly aid in tumor progression or even in the initiation and cancer development. As CMV proteins are mainly confined to metastatic cells in metastases of breast and colon cancer[26,27], CMV may be maintained within cells that initiate the development of the metastasis. However, the role of CMV in tumor initiation, progression and metastasis formation needs to be further thoroughly examined in depth since some researchers have failed to detect CMV in tumors[28-33]. This causes confusion in the field and voices have been raised that the presence of CMV in tumors is false and based on artefact data. However, stimulation of dendritic cells with glioblastoma tumor lysates leads to expansion of CMV specific T cells in glioblastoma patients and CMV pp65 specific T cells can kill autologous glioblastoma cells *in vitro*. This suggests that CMV epitopes are present in glioblastoma tumors[34,35]. The inconsistency in the detection of CMV in tumors samples between laboratories is most likely due to differences in the sample preparations, control specimens and sensitivity of the methods employed for the detection of CMV nucleic acids or proteins. Therefore studies to search for CMV in tumors should be based on techniques that have been developed and proven to work in tumor specimens.

HUMAN MALIGNANT BRAIN TUMORS; GLIOBLASTOMA AND MEDULLOBLASTOMA

Human brain tumors are diverse neoplasms that currently include more than 120 different clinopathological entities[36]. Glioblastomas and medulloblastomas are the most frequently occurring malignant brain tumors in adults and children, respectively. Gliomas accounts for approximately 75% of all primary brain tumors, whereas medulloblastoma is the most common solid childhood tumor[37,38]. The median age of diagnosis of gliomas is 64 years whereas for medulloblastomas about 99% of the tumors are detected in early childhood or adolescence[36,39].

Gliomas are histopathologically classified as ependynomas, astrocytomas, oligodendrogliomas and mixed oligo-astrocytomas and graded with regard to malignancy as Grade Ⅰ-Ⅳ. Grade Ⅲ (anaplastic astrocytomas) and Ⅳ gliomas [glioblastoma (GBM); glioblastoma multiforme] account for approximately 82% of cases and are considered malignant or high-grade gliomas[39]. The current standard of care for malignant gliomas includes surgical resection, radiation therapy with concomitant and adjuvant chemotherapy[40]. Despite aggressive treatment approaches, the median survival for patients diagnosed with Grade Ⅳ glioblastoma is 16 to 19 mo with 25%-30% of the patients alive 2 years after treatment[41].

Malignant gliomas are heterogenous tumors with different clinical and molecular signatures. Glioblastomas have been divided into four molecular subtypes characterized by abnormalities in *PDGFRA*/*IDH1*, *EGFR*, and *NF1* that represent the proneural, classical and mechencymal subtypes, respectively[42]. In addition, a fourth transcriptional subtype termed neural subtype is characterized by high expression of neural markers like *NEFL*, *GABRA1*, *SYT1*, and *SLC12A5*[42]. Many of the molecular abnormalities overlap between the different glioblastoma subtypes and additional rare mutations and chromosomal aberrations have been described, adding to the heterogeneity of glioblastomas. Cells of origin for glioma are not clearly identified but neural stem cells and oligodendrocyte precursor cells that both derive from a neuroepithelial cell, have been suggested[43].

Medulloblastomas are also a compilation of molecular and clinical diverse tumor types that arise either in the cerebellum or brainstem[44]. These tumors are

highly malignant and current treatment of patients with medulloblastoma consists of surgery, whole brain and spinal cord radiation (in patients above 3 years) and aggressive chemotherapy, sometimes followed by stem cell transplantation[44]. Even though long-term survival among medulloblastoma patients is 60%-70%, the patients frequently experience disease or treatment related complications including developmental, neurological, neuroendocrine, and psychosocial deficits[45]. Although medulloblastomas contain significantly less mutations than adult cancers[46], specific subsets have been identified and aberrant expression of key molecules regulating developmental signaling cascades, oncogenic drivers and mutations in tumor suppressor genes, have been described, such as alterations of the Shh and Wnt signaling pathways, overexpression of MYC, MYCN and activation of growth factor independent 1 family proto-oncogenes, GFI1 and GFI1B by enhancer hijacking[44,47-49]. However, since many medulloblastoma tumors have no apparent mutations of any known cancer gene, it has been suggested that epigenetic changes or other etiological factors including infections also may be responsible for tumor initiation and progression[9,44,46,50].

Medulloblastomas have been linked to disordered mechanisms of normal development and medulloblastoma cells retain many features resembling precursor cells of the embryonic brain. Approximately half of these tumors contain abnormal activation of the developmental signalling cascades, Shh and Wnt[51,52]. Moreover, activation of the PI3K/Akt signalling pathway has been shown to be important for initiation and proliferation of medulloblastoma[53-55]. Molecular analysis have shown that there are four major medulloblastoma subgroups (Wnt, Shh, Group 3 and Group 4[44]). These subgroups are distinct in tumor cell histology and biology and exhibit divergent clinical phenotypes. Different subtypes of medulloblastoma are also believed to have distinct cellular origins. One subtype originates from cerebellar granule neural precursor cells located in the external granular layer of the cerebellum as a result of aberrant Shh signalling[56,57]. A subpopulation of cells from these tumors is positive for the progenitor markers Math1 and CD15[58]. A different medulloblastoma subtype arises outside the cerebellum, likely from cells of the dorsal brainstem and is dependent on Wnt signalling. These tumors contain aberrantly proliferating Zic (+) precursor cells[59]. Finally, evidence of a third medulloblastoma subtype deriving from CD133-positive (Prom1) cerebellar stem cells has also been proposed. These tumors contain elevated Myc expression[60,61].

Many malignant gliomas and medulloblastomas are hence thought to initiate from precursors cells within the CNS by sequential and cumulative genetic alterations or developmental errors[43,49,62]. Rare hereditary syndromes including Cowden, Turcot, Li-Fraumeni, tuberous sclerosis, neurofibromatosis and schwannomatosis have been associated with increased risk of glioma, whereas increased risk of medulloblastomas have been observed in individuals with Turcot, Gorlin and Li-Fraumeni syndromes[62]. However, the etiology behind the vast majority of gliomas and medulloblastomas still remain largely unknown and no fundamental environmental factors, except ionizing radiation that is associated with increased risk for glioma development, has been convincingly demonstrated[37]. On the other hand, it seems to be a strong inverse relationship between atopic diseases and glioma[63]. Both glioblastomas and medulloblastomas express high levels of cyclooxygenase-2 (COX-2) that catalyzes conversion of arachidonic acid to prostaglandins and other eicosanoids with concomitant secretion of proinflammatory prostaglandin E_2 (PGE_2)[64-66]. In gliomas, the level of COX-2 expression is directly correlated to glioma grade and associated with shorter survival in glioblastoma patients[65]. Non-steroidal anti-inflammatory drugs, capable of inhibiting cyclooxygenase, significantly suppress the growth of glioblastoma and medulloblastoma in preclinical models[9,64,66]. Also, short-term use (< 10 years) of anti-inflammatory medication is associated with a protective effect against glioblastoma[67]. Taken together, this suggests that brain tumors are at least partly dependent on an inflammatory microenvironment in order to proliferate and progress.

An inflammatory microenvironment can be induced directly by tumor cells through activation of oncogenes that activate transcriptional programs leading to the production of pro-inflammatory eicosanoids, cytokines and chemokines that attract different immunological cells to the surrounding tumor microenvironment. Inflammation in the tumor microenvironment can also be caused indirectly by viral and microbial infections, autoimmune diseases, and dietary products[68]. Tumor-related inflammation is hence important for tumor cells to sustain a proliferative state, escape apoptosis and enhance angiogenesis, metastasis and suppression of the immune system[68].

CMV'S POTENTIAL ROLE IN BRAIN TUMORS: CMV PROTEINS CONFER BOTH ONCOMODULATORY AND ONCOGENIC FUNCTIONS

In the light of the above description of the phenotypic and molecular diversity of gliomas and medulloblastomas, and their different sites of origin in the brain, it is interesting to note that most of glioblastomas and medulloblastomas appear to be CMV positive. The presence of CMV proteins in medulloblastoma and glioblastoma hence raise questions whether this virus plays an important role in tumor initiation and/or progression of these tumors. CMV is not a typical oncogenic virus, but CMV proteins provide many mechanisms that can promote tumor biology relevant mechanisms. During the evolution, there has been a strong evolutionary pressure on CMV to cope with and survive the attacks by the immune system and to create efficient virus factories. CMV was believed to encode for approximately 180 proteins, of which only about 45 have been estimated to be essential for virus replication[69-72]. A more recent study based on ribosomal

profiling suggests that 751 unique CMV proteins are translated in infected cells[73]; if true, this virus is far more complex than previously appreciated. However, regardless of the exact number of CMV proteins that are encoded by this virus, the vast majority of CMV proteins must confer other functions during the virus life cycle than ensuring replication and formation of new virus particles. Several CMV encoded proteins can under certain circumstances initiate cellular transformation or through other ways aid in tumour development and provide mechanisms representing the cornerstones of hallmarks of cancer[74].

The concept of a role of CMV in cancer is not new. Already in the 1970's, Fred Rapp's group reported the frequent presence of CMV in prostate cancer, and isolated a virus strain from tumors that was oncogenic *in vitro* and in immunodeficient mice[75]. However, in several later studies, CMV failed to transform normal human cells, wherefore this virus was not considered to be oncogenic. The classical view implies that oncoviruses encode gene products that can induce cellular transformation under certain circumstances, *e.g.*, HPV, SV40, EBV, Hepatitis B and adenoviruses. For CMV, the term oncomodulation has instead been proposed to describe the indirect influence of CMV on tumorigenesis (reviewed in[6,76,77]). Oncomodulation is defined as the ability to promote, in an appropriate genetic environment supplied by tumor cells, an oncogenic process characterized by disruptions in intracellular signalling pathways, transcription factors and tumor suppressor proteins. For example, CMV proteins control the cell cycle, induce telomerase activity, inhibits apoptosis, induce angiogenesis and cellular migration and hence provide oncomodulatory mechanisms[76-79]. Furthermore, CMV proteins can promote stemness by blocking cellular differentiation and interact with the DNA damage response pathway to alter the cell cycle (reviewed in[77]). CMV proteins induce expression of oncogenes, control expression of tumor supressors, induce specific chromosomal breaks and p53 mutations, inhibit DNA repair mechanisms, control epigenetic functions and cellular proliferation[80-82], and provide immune evasion strategies[83-85].

Experimental data also suggest that CMV can be oncogenic. A gene region of the CMV genome, the transforming region Ⅱ (mtrⅡ), a 980-bp sequence, was first shown to transform rodent fibroblasts[86-90]. Expression of the CMV proteins IE72 or IE86 together with the adenovirus E1A protein can induce cellular transformation through a "hit and run" mechanism[91]. The CMV IE proteins can bind to p53, Rb and degrade p21, and thereby modulate cell cycle regulation, induce telomerase activity[78] and downregulate tumor suppressor proteins, which may aid in oncogenic transformation[77]. The CMV protein US28, a G coupled chemokine receptor homologue, has several characteristics resembling a viral oncoprotein[92-95]. Expression of US28 in NIH3T3 cells renders them tumorigenic upon injection in nude mice[94,95], which involves induced COX-2 expression and VEGF production[95]. Furthermore, transgenic mice expressing US28 only in intestinal epithelial cells developed intestinal adenomas and adenocarcinomas[92], by inhibiting glycogen synthase 3β (GSK-3β) activity, resulting in an accumulation of β-catenin and increased expression of Wnt target genes involved in the control of cell proliferation[92]. US28 also induces STAT3 phosphorylation through IL-6 production, which correlates with poor survival in GBM patients[93]. Analysis of clinical GBM samples *in situ* showed co-localization of US28 with phosphorylated STAT3, COX-2, VEGF and e-NOS, and US28 can induce cellular migration *in vitro*, which suggests that US28 may contribute to tumour invasiveness and angiogenesis *in vivo*[77,93,96]. CMV protein expression in mucoepidermoid cancer also correlated with activation of known oncogenic pathways such as EGFR, ERK and amphiregulin, and protein expression was related to severity[97]. These experimental data suggest a direct molecular link between the expression of US28 and tumorigenesis. In addition, US28 has also been shown to activate the transcription factor nuclear factor B (NF-Kβ), a critical regulator of immunity, stress responses, apoptosis, cellular differentiation and migration[96].

More recently, the microenvironment at the tumor site and the potential close connection to inflammation has received increasing attention, and there seems to be a close link between inflammation and tumor development. COX-2 and PGE_2 are over-expressed in a number of different cancers and high COX-2 expression is often correlated with poor prognosis[66,98]. CMV infection induces COX-2 and 5-lipoxygenase (5-LO) expression and mediate production of PGE_2 and leukotrienes that are both potent inflammatory mediators[99,100]. PGE_2 also induces cellular proliferation, angiogenesis, inhibition of apoptosis and stimulation of invasion, and can contribute to the generation of a tumour promoting inflammatory microenvironment[98]. Interestingly, we observed a clear association between CMV protein expression and COX-2 expression in medulloblastoma, suggesting that CMV may control COX-2 expression in these tumors[9]. Viruses could also by their sole presence induce an immune response through expression of non-self peptides to T cells and create an inflammatory microenvironment.

Epithelial cells can undergo a transition into mesenchymal cells [epithelial to mesenchymal transition (EMT)], involving a series of events resulting in the loss of cell-to-cell contacts and dramatic remodelling of the cytoskeleton. In addition to its role in normal physiological development, recent data implicates a role for EMT and mesenchymal to epithelial transition (EndoMT) in tumor pathology, particularly in regards to metastatic capacity of epithelial tumors. A major factor that regulates the EMT process is transforming growth factor beta (TGFβ). CMVs ability to induce TGFβ provides a role of this virus to facilitate the EMT process[101]. In support of this hypothesis, CMV infected epithelial cells treated with TGFβ *in vitro*, were shown to undergo morphologic and transcriptional changes similar of EMT; this also occurred in uninfected cells[102]. CMV infected epithelial cells can also activate extracellular latent TGFβ1 through induction of metalloproteinase 2 (MMP-2), which was proposed to be mediated by the CMV proteins CMV IE72 or IE86[102]. Induced MMP-2 activity could also in theory mediate

degradation of the extra cellular matrix[103], which would further aid in the formation of a metastasis. In addition, CMV US28 can interfere with the activity of expression of GSK3β, which is known to phosphorylate and control the stability of key oncogenic transcription factors such as the Smads and Snail that can trigger an EMT program. Furthermore, virus induced COX-2 expression, and activation of Ras/Erk and PI3K/AKT signalling pathways may further induce and maintain a viscous paracrine loop leading to possible cellular invasion into surrounding stroma.

POTENTIAL TREATMENT OPTIONS TARGETING CMV IN BRAIN TUMORS

We found CMV DNA and proteins in 92% of primary medulloblastoma tumors and in 99% of glioblastomas and also detected the virus in eight of eight examined medulloblastoma cell lines, grown in culture for decades[9]. When the medulloblastoma cells were implanted subcutaneously in immunodeficient mice, all tumors were CMV protein positive; a majority of the tumor cells expressed CMV IE proteins[9]. We found CMV proteins in medulloblastoma cells in culture expressing CD133 and CD15, which are proposed markers of medulloblastoma stem cells. The cancer stem cell hypothesis states that only a subpopulation of cancer cells have self-renewing ability and the capacity to give rise to tumours[104]. Such cancer stem cells or tumour initiating cells (TICs) exhibit an immature phenotype, *e.g.*, expression of pluri-/multipotency associated transcription factors[105], a slower proliferation rate and increased resistance to cancer therapy relative to more differentiated cancer cells[106]. In theory, TICs could either be directly infected by CMV as immature cells or represent a dedifferentiated mature cell with a potential ability to affect tumorigenesis and EMT. CMV infected tumor cells undergoing EMT may detach from adjacent cells and potentially enter the circulation *via* the lymphatic system or the blood stream. It is possible that CMV positive tumor cells in primary breast, prostate and colon tumors can undergo EMT to obtain stem cell characteristics, *i.e.*, a potential TIC/ EMT cell will circulate, undergo the reverse process of EndoMT as metastases are developing in lymph nodes or distant organs such as the brain. If CMV resides in TICs, it would explain why all xenografted tumors were virus positive, although only a minority of medulloblastoma tumor cells in culture expressed CMV proteins[9]. If this holds true, drugs targeting CMV infection may not only be beneficial to inhibit primary tumor growth but may also reduce the capacity of the tumour to become invasive as it may selectively kill the TICs. Such scenario implies that most stages of tumor development, primary growth, migration, invasion, intravasation and potentially metastasis may be sensitive to anti-viral drugs making CMV an ideal target for therapeutic intervention.

Hence, regardless if CMV plays a role in the development of tumors, CMV has been detected in brain tumors such as glioblastoma and medulloblastoma and virus infected tumor cells may therefore represent a new target of therapy. In support of this hypothesis, we showed that animals carrying CMV positive human medulloblastoma or neuroblastoma tumors that were treated with anti-CMV drugs had significantly smaller tumors than placebo treated animals[9,107]. A synergistic effect was observed with a COX-2 inhibitor, which resulted in a 72%-97% reduced medulloblastoma tumor growth *in vitro* and *in vivo*[9]. Both COX-1 and COX-2 inhibitors are efficient anti-viral drugs that prevent the replication of CMV[99,108]. Thus, antiviral drugs and COX-2 inhibitors may act synergistically to affect the growth of CMV positive tumors[9]. Interestingly, a number of recent studies demonstrate that Aspirin, a non-selective COX- inhibitor, significantly prevents cancer development and metastases[109-113]. With current data at hand, it cannot be excluded that some of the preventive effect of COX-2 inhibitors involves CMV mediated tumor mechanisms.

Treatment of GBM xenograft tumors with the anti-CMV drug Cidofovir also reduced tumor growth, although a CMV independent mechanism was observed[114]. Importantly, treatment of 50 glioblastoma patients who received anti-CMV treatment as an add-on to standard therapy at Karolinska University Hospital as adjuvant treatment demonstrate a remarkably high survival: the 2 year survival was 70% among 40 patients receiving 6 mo of anti-viral therapy and as high as 90% among patients with continuous treatment (n = 25) compared with 18% in contemporary controls (n = 137); median OS was 56.4 mo compared with 13.5 mo in controls in the latter group ($P < 0.0001$[115]). These observations call for a deeper understanding of CMVs role in cancer and whether this virus is a novel target in anti-cancer therapy. Also, anti-CMV drugs used as an add-on therapy should be further analysed in larger glioblastoma patient populations to give robust statistical data in randomised trials to confirm or dismiss the use of valganciclovir in these patients. The presence of CMV in glioblastoma would also imply that immunotherapy protocols that target CMV epitopes expressed in the tumor can be exploited as cancer therapy[116-118]. Several immunotherapy protocols are currently under evaluation in clinical trials to evaluate different CMV based protocols for glioblastoma patients; these need to also consider the immunosuppressive state of glioblastoma patients.

Several studies indicate that GBM patients exhibit functional impairments of their T cell functions[119,120]. However, polyfunctional CMV specific T cells can be restored by *in vitro* stimulation with CMV antigens and gamma C cytokines. It was recently demonstrated the CMV pp65 specific T cells can kill autologous glioblastoma cells *in vitro*[35], and that immunotherapy using CMV specific T cells was associated with prolonged survival in a single patient[18]. This suggests that adoptive therapy of *in vitro* expanded T cells may be a preferred protocol to be used to overcome the problem of unresponsive T cells *in vivo*, although T cell activation may be possible to overcome by the right stimulus also *in vivo*. Today

two clinical studies are open for enrolment of GBM patients; one is testing genetically modified CMV specific cytotoxic cells for recurrent GBM; a chimeric antigen receptor recognizes human epidermal growth factor receptor 2 coupled to CD28. The other is based on DC vaccination together with a monoclonal antibody against CD25 aimed to inhibit IL-2 signalling. Results from these trials are expected in 2015 and 2016, respectively, and currently results from two other studies that are closed for recruitment are also awaited in the near future. Most likely, future protocols will have to evaluate the effect of immunotherapy in combination with anti-viral therapy for CMV to obtain optimal anti-CMV effects in cancer patients.

CONCLUSION

In summary, emerging data suggest that CMV may play a pathogenic role in cancers of epithelial and neuronal origin. Under such circumstances, anti-viral treatment strategies may provide new options in cancer therapy of CMV positive tumors and metastases to improve patient outcome.

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REVIEW

Internal ribosome entry site-based vectors for combined gene therapy

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Abstract

Gene therapy appears as a promising strategy to treat incurable diseases. In particular, combined gene therapy has shown improved therapeutic efficiency. Internal ribosome entry sites (IRESs), RNA elements naturally present in the 5′ untranslated regions of a few mRNAs, constitute a powerful tool to co-express several genes of interest. IRESs are translational enhancers allowing the translational machinery to start protein synthesis by internal initiation. This feature allowed the design of multi-cistronic vectors expressing several genes from a single mRNA. IRESs exhibit tissue specificity, and drive translation in stress conditions when the global cell translation is blocked, which renders them useful for gene transfer in hypoxic conditions occurring in ischemic diseases and cancer. IRES-based viral and non viral vectors have been used successfully in preclinical and clinical assays of combined gene therapy and resulted in therapeutic benefits for various pathologies including cancers, cardiovascular diseases and degenerative diseases.

Key words: Vector; Gene transfer; Internal ribosome entry site; Gene therapy

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Core tip: Combined gene therapy has emerged for a few years as a promising strategy to improve treatments of many diseases including cancer, cardiovascular diseases and degenerative diseases. In this context, internal ribosome entry site (IRES)-based vectors provide a powerful system to co-express several therapeutic genes from the same transcription unit. IRESs are translational enhancers, exhibiting tissue-specificity, and activated by stress. Different IRES-based vectors including plasmids, adeno-associated virus-derived and lentiviral vectors have been used successfully in many preclinical protocols of gene therapy. Moreover the few clinical assays launched with IRES-based multicistronic vectors resulted in therapeutic benefits.

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INTRODUCTION

Combined gene therapy has appeared for a few years as an attractive approach to optimize the therapeutic benefits of gene transfer. In the field of cancer, the first examples of antitumoral cooperative effect have been provided by co-expression of the co-stimulation molecules CD70 and CD80, and of the two anti-angiogenic factors, angiostatin and endostatin, respectively[1-4]. Synergistical effects have also been obtained with co-expression of angiogenic growth factors generating therapeutic angiogenesis in ischemic diseases. This rational has been proven first with co-administration of vascular endothelial growth factor A (VEGFA) and angiopoietin as recombinant proteins as well as by co-administration of two plasmids coding these growth factors[5]. A few years later, combination of recombinant fibroblast growth factor 2 (FGF2) and PDGF-B also improved hindlimb ischemia in rats whereas a bicistronic vector expressing FGF2 and VEGFA efficiently induced vessel formation in a mouse angiogenesis assay[6,7]. These studies launched the concept of combined biotherapy. They also revealed that combined gene therapy is a promising therapeutic approach, allowing long term efficiency of treatments compared to recombinant proteins whose half life is often very short.

Internal ribosome entry sites (IRESs) are translational enhancers naturally present in a series of mRNAs, mediating internal initiation of translation when present between the genes of interest (Figure 1). IRESs thus allow the design of multicistronic expression cassettes resembling bacterial operons, able to drive translation of several genes coded by the same mRNA[8]. We have demonstrated that the use of IRES-based vectors co-expressing two genes of interest allows stable transgene expression with a constant ratio of the proteins of interest, in contrast to the use of two different plasmids expressing each transgene[9]. Actually, a bicistronic IRES-based vector co-expressing FGF2 and Cyr61 has revealed more efficient to generate therapeutic angiogenesis at low doses than the monocistronic vectors expressing large amounts of only one of these angiogenic factors[10]. It must be underlined that the IRES-based vector had no side effects on promotion of tumoral angiogenesis in contrast to the monocistronic ones, a very important feature for increased safety in clinical assays. These observations prompted us to deepen the features of IRESs applicable to vectorology and assess progress made in the field of gene transfer and combined gene therapy clinical assays using IRES-based vectors.

IRESS, TRANSLATIONAL ACTIVATORS FOR COMBINED TRANSGENE EXPRESSION

At a time when it was admitted that initiation of translation in eukaryotes required recognition of the capped mRNA 5' end to recruit ribosomes, translation of the uncapped picornavirus mRNAs from an internal start codon remained a mystery. Indeed, the so-called ribosome scanning mechanism predicted that ribosomes bound to the mRNA 5' end scanned the mRNA molecules until they recognized an AUG codon[11,12] (Figure 1). The event of internal ribosome binding was thought impossible. This puzzle raised by picornaviruses was solved by the discovery of RNA elements, called IRES, present in the 5' untranslated regions of their mRNAs, which allow internal recruitment of ribosomes[13,14]. The dogma of the scanning mechanism was thus broken. In addition, it was quickly extended to cellular mRNAs as the first cellular IRES was discovered three years later in the BiP mRNA, coding for the immunoglobulin chaperone also known as GRP78[15]. This discovery was followed by the finding of several other IRESs in cellular mRNAs, in particular in the mRNAs of angiogenic growth factors such as FGF2, proto-oncogenes such as c-myc, pro and anti-apoptotic proteins such as X chromosome-linked inhibitor-of-apoptosis protein and apoptotic peptidase activating factor 1[16-20]. IRESs were also found in retroviruses, whose mRNAs are capped as cellular mRNAs, leading to the design of IRES-containing retroviral vectors[21,22].

The existence of IRESs in capped cellular mRNAs asked the question of their pathophysiological function[23]. Actually, several reports showed that IRESs from cellular mRNAs are regulated in various physiological processes including cell differentiation, spermatogenesis, neurone plasticity[24-27]. Several IRESs are also activated during cell cycle mitosis[28,29]. Recent reports have also shown that IRESs are aberrantly activated in tumor cells, and are thus involved in dysregulation of gene expression in cancer[30]. Furthermore, cellular IRES activity is stimulated in stress conditions such as apoptosis and hypoxia when cap-dependent translation is blocked[31-36].

IRES-dependent internal initiation of translation reminds the prokaryotic initiation mechanism which can translate polycistronic mRNAs[37,38]. This observation gave the idea that such operons could be created in eukaryotes using IRESs to design expression vectors[39]. A large majority of expression vectors allow co-expression of two genes under the control of two promoters. However such an approach has revealed that one of the genes may be silenced despite of the expression of the other one even though it expresses an antibiotic[40]. This can result from competition between the two promoters or counterselection of the gene of interest in case of toxicity or of cell growth inhibition. In such a context, IRESs have been used to generate transgene co-expression under the control of a single promoter (Figure 2).

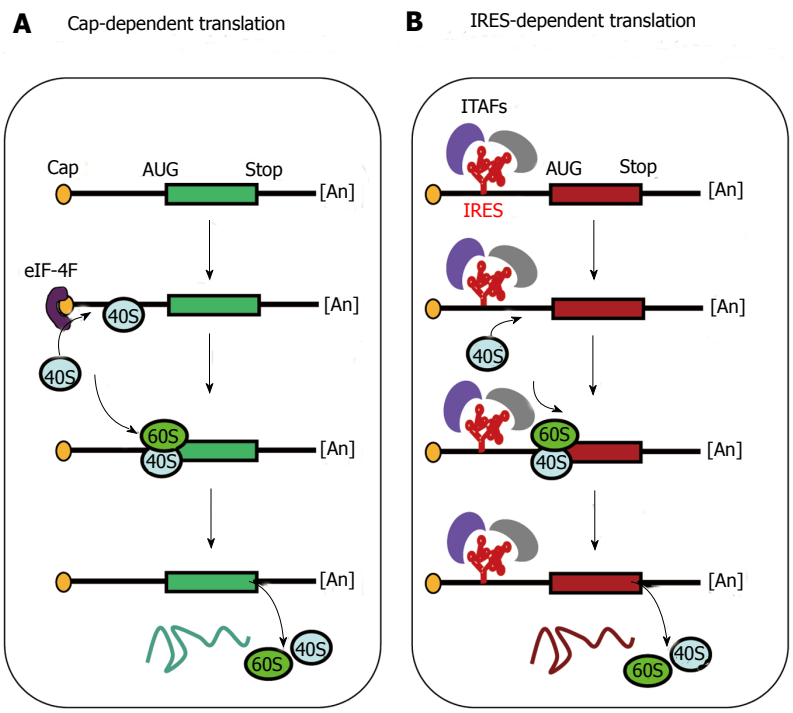

Figure 1 Cap-dependent and internal ribosome entry site-dependent initiation, two alternative mechanisms of translation. A: The so-called cap-dependent ribosome scanning mechanism predicts that ribosome 40S subunit binds to the mRNA 5' end. Ribosome binding requires the initiation factor 4F (eIF-4F, composed of the three proteins eIF-4E, -4A and -4G). Then the mRNA is unwound under the control of the helicases eIF-4A and -4B, allowing the ribosome to scan the mRNA until recognition of an initiation codon (classically AUG)[11,12]; B: When an Internal ribosome entry site (IRES) is present in the mRNA 5' untranslated region, IRES trans-acting factors (ITAFs) allow ribosome 40S internal recruitment, independently of the presence of cap and eIF-4F. The IRES-dependent mechanism occurs in the case of picornavirus uncapped mRNAs as well as for cellular capped mRNAs.

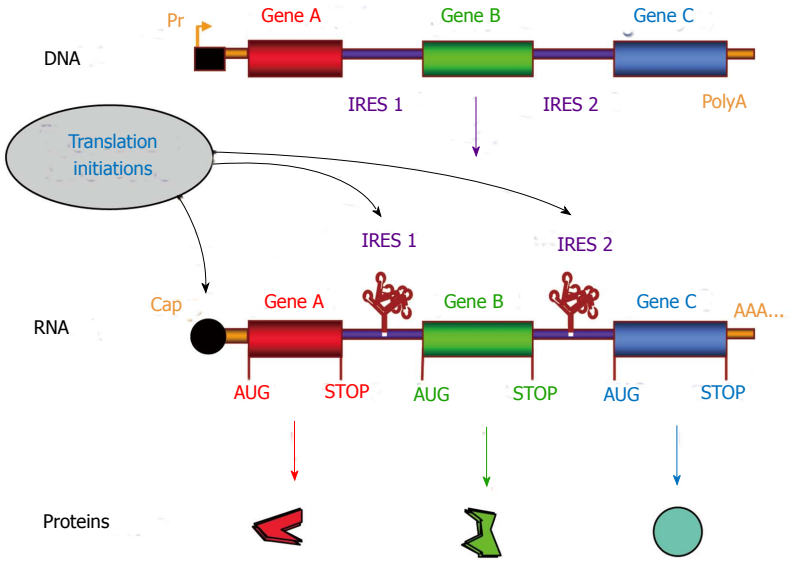

Figure 2 Internal ribosome entry site-based multicistronic vector concept. The internal ribosome entry site (IRES)-based expression cassette contains several genes, separated by IRESs, under the control of the same promoter (Pr). This transcription unit gives rise to a single mRNA coding the different genes. Translation initiation occurs at the 5' end by the cap-dependent mechanism, resulting in translation of the first open reading frame (ORF, Gene *A*). Internal initiations of translation occur at each IRES, resulting in translation of the other ORFs (Genes *B* and *C*). Thus the multicistronic mRNA generates several proteins from a single transcription unit, allowing more stable long term expression and stable transgene ratio[9,48]. For each ORF, initiation (AUG) and termination (STOP) codons are indicated.

The first retroviral tricistronic IRES-based vector appeared in 1992, providing an exciting potential for gene therapy[41]. This vector successfully co-expressed adenosine desaminase with neomycin (NEO) resistance and chloramphenicol acetyltransferase reporter genes, using the two picornavirus IRESs from poliovirus and encephalomyocarditis virus (EMCV), respectively. Two years later, a therapeutic tricistronic vector expressing the two interleukin-12 subunits with NEO validated the concept of IRES-based vectors to co-express two subunits of a protein with an adequate stoechiometry together with a resistance gene[42]. In the following years, bicistronic vectors were used successfully to select cell clones expressing a protein of interest with a resistance gene, preventing the problems generated by the use of two promoters[40,43].

TISSUE-SPECIFICITY OF CELLULAR IRESS

Most IRES-based vectors developed up to now use picornavirus IRESs, based on the strong efficiency of such IRESs in transient transfection, compared to cellular IRESs. It has been observed that cellular IRESs often exhibit a low efficiency in transiently transfected cells. Such a feature may result from the cell and tissue specificity of the cellular IRES activities. Actually, the FGF2 IRES activity varies with the cell type, the lowest being in fibroblasts, and the highest in neuroblastoma and osteosarcoma cells[44]. Similar variations have been observed for other cellular IRESs[45] (Creancier L and Prats AC, unpublished results). The strongest regulation

of cellular IRESs has been shown *in vivo*, in transgenic mice expressing bicistronic dual luciferase constructs containing different IRESs. Clearly, the EMCV IRES was active in most tissues and organs, while the FGF2 IRES was very low in most organs except for testis and brain where its activity increased 200 to 400 times, at least 10 times higher than the EMCV IRES activity[44]. A similar behavior was observed with other cellular IRESs such as c-myc and VEGFA IRESs[31,45].

The tissue-specific features of cellular IRESs are useful to control transgene expression. Thus they can be considered as translational enhancers, if one makes a parallel with transcriptional enhancers upstream of promoters, governing the tissue-specificity of gene expression. The concept of translational tissue-specificity may be applied to gene transfer by coupling tissue-specific IRESs with tissue-specific promoters to create vectors with increased safety. This concept should also remember us that EMCV is not always the best IRES to be used. A recent study reports the failure of expression of the second cistron of a bicistronic adeno-associated virus (AAV) vector using the EMCV IRES, in murine cerebellar Purkinje neurons[46].

The advantage of using a cellular IRES has also been demonstrated for gene transfer into skeletal muscle. The FGF1 IRES is as efficient as the EMCV IRES in mouse muscle after plasmid DNA electrotransfer[47]. Moreover, when this IRES is used in a bicistronic AAV vector, its activity is significantly superior to that of the EMCV IRES in myoblasts and allows a transgene expression 10 times more efficient when this AAV is injected in mouse muscle[48]. Such a difference may be due to the presence of specific FGF1 IRES trans-acting factors (ITAFs) (Ainaoui *et al*, in revision). Alternatively, it can result from the lower ability of the EMCV IRES to maintain a stable long term compared to cellular IRESs, shown in a previous report[9].

On the basis of these different data, it can be recommended to choose the adequate IRES to be used according to the cell type or tissue to be targeted, rather than using systematically the EMCV IRES as presently proposed in all commercial IRES-based vectors.

IRES-MEDIATED GENE EXPRESSION IN STRESS CONDITIONS

In many diseases cells are subjected to different stresses such as hypoxia, apoptosis or ER stress. In stress conditions, translation initiation is inhibited by two ways: blockade the mammalian target of rapamycin pathway which affects ribosome recruitment on the cap, and phosphorylation of eIF2-α which prevents charged initiator Met-tRNA formation. Interestingly, IRES-dependent translation is not affected by these two ways of silencing[35,49,50].

As mentioned above, IRESs are naturally present in messenger RNAs coding for proteins involved in the stress response, especially apoptosis and hypoxia. In particular, an IRES is present in the mRNA of the hypoxia-induced factor 1α (HIF1α), the key of the cell response to hypoxia that induces transcription of all the genes containing a hypoxia responsive element (HRE) in their promoters[51]. This IRES allows HIF mRNA translation to be activated during hypoxia despite of the blockade of global translation[32,52]. Such activation occurs under the control of an ITAF, the pyrimidine tract binding protein, also known as a regulator for various IRESs[52,53].

An important consequence of hypoxia is the stimulation of angiogenesis in order to generate new vessels able to restore the cell supply with oxygen. This process occurs in cancers when cells in the tumor core are oxygen deprivated, as well as in ischemic diseases such as heart and lower limb ischemia when tissues are not any more irrigated due to artery occlusion. Strikingly, the major angiogenic factors VEGFA (vascular endothelial growth factor A), FGF1 and FGF2, possess IRESs in their mRNAs[20,47,54-56]. VEGFA expression, transcriptionally induced by HIF1α, is also translationally enhanced *via* the IRES in hypoxic tumors and in ischemic mouse legs[31,32,36]. In contrast to VEGFA, FGF2 is not induced transcriptionally by hypoxia but its synthesis is translationally induced by the IRES-dependent mechanism in ischemic tissues[31,33]. The same phenomenon has been observed for the major lymphangiogenic factor VEGFC, induced by hypoxia at the translational level *via* an IRES, but not at the transcriptional level, in tumors and lymph nodes[36,57]. FGF2 and VEGFC induction is exclusively translational and HIF-independent, revealing that IRESs provide an alternative HIF-independent way of response to hypoxia.

On a biotechnological point of view, the sensitivity of IRESs to hypoxia may be an advantage for several applications. Gene transfer vectors can benefit from this feature as the presence of IRESs allows increased transgene expression in ischemic conditions *in vivo*. Once again, one can see that data from basic research have to be taken into account in the design of optimized expression cassettes. The use of IRES-based vectors seems particularly adequate for gene therapy of ischemic diseases and cancer, as in both cases the transgenes have to be expressed in hypoxic conditions.

BIOMEDICAL APPLICATIONS OF IRESS

IRESs have found biomedical applications for several years. As mentioned above, the first biomedical use of IRESs in an expression vector has been co-expression of subunits of a therapeutic protein with a gene of resistance, as shown for interleukin 12 subunits with a gene of resistance[42]. However this application is limited to therapeutic genes composed of several subunits. In addition, the use of resistance genes is not recommended as it may prevent the use of the vector in a clinical assay.

Another application of IRESs raised during the last decade, resulting form the emerging concept of combined gene therapy. Several studies have validated this concept using a cocktail of two vectors to transfer two genes simultaneously. This has been particularly

Table 1 Preclinical studies of combined gene therapy with co-administration of monocistronic vectors

Pathology	Therapeutic genes	Animal model	Vector type	Ref.
Cancers				
Leukemia, melanoma	Angiostatin + endostatin	Mouse	Retrovirus	Scappaticci *et al*[4], 2001
Ovarian cancer	Angiostatin + endostatin	Mouse	AAV	Ponnazhagan *et al*[3], 2004
Glioblastoma	*VEGF-R1* + angio-endo (Statin AE)	Mouse	SB transposon	Ohlfest *et al*[60], 2005
Pancreatic cancer	*TSP1* + endostatin	Mouse	AAV	Zhang *et al*[61], 2007
Cardiovascular diseases				
Limb ischemia	*VEGFA* + angiopoietin-1	Rabbit	Plasmid	Chae *et al*[5], 2000
Limb ischemia	*VEGFA* + *FGF2*	Mouse	Plasmid	Lee *et al*[59], 2007
Limb ischemia	*VEGFA* + *PDGFB*	Rabbit	AAV	Kupatt *et al*[58], 2010
Heart ischemia	*VEGFA* + *PDGFB*	Pig	AAV	Kupatt *et al*[58], 2010
Rear diseases				
DMD	Microdystrophin + IGF1	Mouse	AAV	Abmayr *et al*[62], 2005

DMD: Duchenne muscular dystrophy; VEGF: Vascular endothelial growth factor; FGF2: Fibroblast growth factor 2; AAV: Adeno-associated virus.

documented in the field of cardiovascular diseases and cancer, with therapeutic benefits obtained in different animal models using different combinations of angiogenic or anti-angiogenic factors[4,5,58-61] (Table 1). Interestingly the combination of VEGFA and PDGFB successfully induced therapeutic angiogenesis both in ischemic leg and in ischemic heart. In the field of rare diseases, two AAV vectors expressing microdystrophin and IGF1 resulted in increased muscle mass and strength, reduced myofiber degeneration and increased protection against contraction-induced injury in *mdx* mice[62]. These different studies were performed either with naked DNA or with recombinant adeno-associated virus vectors.

The use of two different vectors for multiple transgene expression exhibits disadvantages: on the one hand, the ratio of the therapeutic molecules cannot be controlled, leading in the loss of the cooperative effect: expression of one of the vectors often decrease or is silenced earlier than the other one[40]. On the other hand, the cost of two therapeutic vectors in a clinical perspective is higher than a single one. These disadvantages are still more important in case of a cocktail of three or more therapeutic genes.

The concept of IRES-based vectors for combined gene therapy has been validated for combined immunotherapy of cancer using a tricistronic retrovirus expressing the two co-stimulation molecules CD70 and CD80[2] (Table 2). In addition to the EMCV IRES, several cellular or retroviral IRESs were successful in this approach[63]. *In vivo* gene therapy has also been validated for the treatment of ischemic limb in a mouse model, following intramuscular injection and electrotransfer of a plasmid containing the FGF1 IRES for co-expression of FGF2 and Cyr61[10]. This study showed than the two angiogenic factors, although expressed at lower doses from the bicistronic vector than from the monocistronic ones, have a synergistical effect in stimulating therapeutic angiogenesis, rendering the bicistronic construct more efficient. More importantly, due to the lower doses of therapeutic molecules, the bicistronic vector induces no side effects on tumoral angiogenesis, in contrast to one of the monocistronic vectors expressing huge amounts of Cyr61. Thus combined gene therapy using IRES-based vectors is also a safer therapeutic approach.

Additional studies have confirmed the successful use of IRES-based vectors for combined treatment of limb ischemia with VEGFA and FGF4 or bone morphogenetic protein7 (BMP7)[64,65]. Combined gene therapy of cancer was also reported using IRES-based vectors co-expressing IL-12 and CD80, as well as antiangiogenic factors angiostatin and endostatin, or CXCL4I and fibstatin[66-69] (Table 2). Combination of angiostatin and endostatin in an IRES-based vector was also successful to treat age-related macular degeneration in a mouse model[70]. In the field of degenerative diseases, mucopolysaccharidosis type ⅢA has been addressed in presymptomatic MPSⅢA mice by intrastriatal administration of an AAV vector co-expressing N-sulfoglycosamine sulfohydrase (SGSH) with the sulfatase-modifying factor (SUMF1) (Winner *et al*, submitted). This study has resulted in a clinical assay[71] see below). Only one report has obtained better data with two separate AAV vectors to deliver FGF14 and a fluorescent protein into *purkinje* neurons, than with an IRES[46]. This study used the EMCV IRES previously reported to function in neurons[72]. However it must be underlined that the EMCV IRES is not very active in neurons *in vivo*, by comparison with the FGF2 IRES that is at least ten times more active[24,44]. In such a case, one can expect that the choice of the FGF2 IRES would provide better data.

Multigene transfer has also been validated for combinations of three genes. A tricistronic IRES-based lentivector expressing three catecholaminergic proteins, Prosavin, was administrated by bilateral striatal injection for treatment of Parkinson in rats, resulting in important therapeutic benefits[73,74] (Table 2). Moreover, a tricistronic 2A-based lentivector administrated in situ was also efficient in co-expressing Gata4, Mef2c and Tbx5 for postinfarct ventricular functional improvement in rats[75].

It is often mentioned that the IRES-driven translation of the downstream cistrons is lower than the cap-dependent first cistron translation. This issue can easily be addressed by intelligent vector design: First, one can take into account the tissue specificity of the IRES by choosing the most adequate IRES rather than using systematically the EMCV IRES. Most bi- and- tricistronic vectors use this IRES although it is far to be the best one

Table 2 Preclinical studies of combined gene therapy using multicistronic vectors

Pathology	Therapeutic genes	Animal model	IRES	Vector type	Ref.
Cancers					
Fibrosarcoma	CD70 + CD80	Mouse	EMCV	Retrovirus	Couderc *et al*[2], 1998
Melanoma	Angio-endo fusion	Mouse	None (fusion)	Retrovirus	Scappaticci *et al*[79], 2001
Multiple myeloma	IL12 subunits + CD80	Mouse	EMCV + FMDV	Retrovirus	Wen *et al*[69], 2001; Li *et al*[67], 2003
Melanoma	CD70 + CD80	Mouse	EMCV, c-myc, FGF2, HTLV1	Retrovirus	Douin *et al*[63], 2004
Ovarian cancer	Angiostatin + endostatin	Mouse	EMCV	AAV	Isayeva *et al*[66], 2005
Head and neck cancer	Angio-endo fusion	Mouse	None (fusion)	Vaccinia virus	Tysome *et al*[80], 2011
Pancreas cancer	CXCL4L1 + fibstatin	Mouse	FGF1	AAV, Lentivector	Prats *et al*[68], 2013
Cardiovascular diseases					
Limb ischemia	*FGF2 + Cyr 61*	Mouse	FGF1	Plasmid	Rayssac *et al*[10], 2009
Limb ischemia	*VEGFA + BMP7*	Rabbit	EMCV	AAV	Zhang *et al*[65], 2010
Limb ischemia	*VEGFA + FGF4*	Mouse	EMCV	AAV	Jazwa *et al*[64], 2013
Heart ischemia	*Gata4 + Mef2C + Tbx5*	Rat	None (2A element)	Lentivector	Mathison *et al*[75], 2014
Neurodegenerative diseases					
Parkinson	*TH + AADC + CH1*	Rat	EMCV	Lentivector	Azzouz *et al*[73], 2002 Stewart *et al*[74], 2011
AMD	Angiostatin + endostatin	Mouse	EMCV	Lentivector	Kachi *et al*[70], 2009

BMP7: Bone morphogenetic protein 7; Gata4: GATA binding protein 4; Mef2C: Myocyte-specific enhancer factor 2C; Tbx5: T-box transcription factor 5; TH: Tyrosine hydroxylase; AADC: aromatic L-amino acid decarboxylase; CH1: GTP cyclohydrolase-1; AMD: Age-related macular degeneration.

in many tissues such as muscle or brain[24,48]. Second, the IRES efficiency can be improved. It must be noticed that the EMCV IRES activity is very sensitive to the position of the start codon of the gene of interest. This IRES, in contrast to the FGF1 IRES, exhibits no flexibility: the AUG must be positioned just downstream from the IRES. The insertion of a single restriction site between the IRES and the AUG codon is sufficient to inactivate the IRES[76]. The insertion of a spacer between the first gene and the IRES is also susceptible to enhance the IRES activity by preventing IRES structural alterations by RNA sequences located upstream[77]. In addition, mutations of the upstream AUG codons in the EMCV IRES improve its efficiency[78]. Finally, an important parameter is the IRES regulation by microenvironment. In particular, FGF or VEGF IRES activities are more sensitive to hypoxia than the EMCV IRES and may allow a more efficient transgene expression in ischemic diseases.

ALTERNATIVES TO IRESS FOR MULTICISTRONIC VECTORS

IRES-based vectors are not the only approach to co-express several gene products under the control of a single promoter. The first alternative is gene fusion. It has been successfully used to combine endostatin and angiostatin in a treatment of melanoma and of head and neck cancer[79,80]. A second alternative to IRESs is the use of alternative splicing-based vectors. Such an approach had been proposed many years ago using retroviral vectors, using the natural alternative splicing features of retrovirus genome[81,82]. This concept has been developed more recently in the purpose of co-expressing two immunoglobulin chains[83]. The interest of this system is the ability to adapt the ratio of the two transgenes by mutating the splicing sites. However one limit of this attractive system is that splicing site efficiency and consequently the ratio of the two proteins of interest, is influenced by the presence of exon splicing enhancers or silencers in the transgene sequences, preventing the design of vectors with a stable transgene ratio applicable to co-expression of any pair of therapeutic proteins.

A third exciting system of co-expression is provided by the 2A peptides. Such peptides, occurring in many viral genomes, are peptide sequences of about 19 amino-acid residues, which can produce a discontinuity in the translated polypeptide when encoded in a longer open reading frame (ORF)[84]. In contrast to what is currently admitted, 2A peptides do not catalyze a protein cleavage, but they catalyze termination of translation in the absence of a stop codon, followed by reinitiation. They are currently used as a tool to co-express two or more separate proteins from a single ORF[85]. 2A peptides thus constitute an alternative to IRESs, but do not work in all systems. By example, in the study in *purkinje* neurons mentioned above, a 2A peptide was used but did not function, resulting in detection of the longer ORF rather than the two expected proteins[46]. In another report comparing bicistronic constructs expressing Sox9 and EGFP separated by the EMCV IRES or by the FMDV 2A peptide, the authors detected 42% of Sox-EGFP fusion protein, reflecting an inefficient ribosome skipping mechanism[86]. Formation of such fusion proteins often occurs with proteins bearing N-terminal signal sequences[87]. In addition, no information is available about the 2A peptides tissue-specificity or behavior in response to stress, in contrast to IRESs.

CLINICAL APPLICATIONS OF IRES-BASED VECTORS TO GENE THERAPY

All the preclinical studies mentioned above show that

Table 3 Clinical studies of combined gene therapy

Pathology	Therapeutic genes	IRES	Vector type	Outcome	Ref.
Ischemic heart disease	*VEGFA + FGF2*	EMCV	Plasmid	Moderate benefits	Kukula *et al*[90], 2011
Parkinson	*TH + AADC + CH1*	EMCV	Lentivector	Benefits for 15/15 patients	Palfi *et al*[91], 2014
Mucopolysaccharidosis type IIIA	*SGSH + SUMF1*	EMCV	Lentivector	Benefits for 1/4 patients, stabilization for 3/4	Tardieu *et al*[71], 2014

TH: Tyrosine hydroxylase; AADC: Aromatic L-amino acid decarboxylase; CH1: GTP cyclohydrolase-1; SGSH: N-sulfoglycosamine sulfohydrolase; SUMF1: Sulfatase-modifying factor.

IRES-based vectors represent an exciting tool to be used for combined gene therapy. Nowadays, very little clinical trials with such vectors have been reported. The first trial to be cited is the tricistronic IL12-expressing retrovirus, which gave significant decrease of tumor sizes on a few patients with melanoma or head and neck cancer[88,89].

A bicistronic IRES-based vector co-expressing FGF2 and VEGFA has been assessed in a clinical assay of gene therapy on patients with refractory coronary disease[90] (Table 3). The protocol corresponded to intramyocardial transfer of a plasmid expressing the bicistronic cassette. This study showed no improvement in myocardial perfusion, but treated patients exhibited improved exercice tolerance and clinical symptoms. Furthermore the bicistronic gene transfer was safe. This moderate benefit, although encouraging, may be due to the use of a plasmid, which does not provide long term expression in contrast to viral vectors, and also to the choice of the EMCV IRES which is not optimal to drive gene expression in hypoxic conditions[31,36].

Very recently, two gene therapy clinical trials successfully used multi-cistronic IRES-based viral vectors. On the one hand, a gene therapy Ⅰ/Ⅱ phase clinical trial on patients with mucopolysaccharidosis type ⅢA, a severe degenerative disease, has displayed neurocognitive benefits[71]. Four children received intracerebral injections of a bicistronic AAV vector expressing the SGSH and SUMF1 genes separated by the EMCV IRES. Neurocognitive evaluations suggest a cognitive benefit on the youngest patient, where as the other ones are stabilized. Importantly, the treatment was safe and well tolerated after 1 year in all the patients, validating the surgical approach for direct AAV delivery in the brain parenchyma. On the other hand, a phase Ⅰ/Ⅱ assay was performed on 15 patients with Parkinson's disease using Prosavin (see above), a tricistronic lentivector with EMCV IRESs administrated by intrastriatal delivery[91]. A significant improvement of motor scores was recorded in all patients at 6 mo. This is the first-in-man use of a lentiviral-based gene therapy vector for a neurodegenerative disease. These studies validate the clinical use of IRES-based viral vectors.

CONCLUSION

Many reports have shown that combined gene therapy is an attractive approach in animal models. This observation has justified extensive research on optimization of gene transfer vectors able to co-express several proteins. In this context, IRES-based vectors have now been validated in pre-clinical as well as in clinical studies by showing their safety and ability to generate therapeutic benefits.

In addition, the data available on IRES tissue-specificity and activation in response to stress provide promising perspectives of vector improvement, which may result in better efficiency of gene therapy.

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REVIEW

Consolidated and emerging inflammatory markers in coronary artery disease

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Abstract

Coronary artery disease is an event of atherosclerosis characterized by a chronic vascular inflammation. Risk factors like obesity, diabetes mellitus, hypertension, smoking, hypercholesterolemia and positive family history sometimes are not sufficiently adequate to the enhancement of cardiovascular risk assessment. In the past years numerous biomarkers, like C reactive protein, cytokines and adhesion molecules, have been observed to be related to adverse cardiovascular prognosis. Recently, several studies found an association among inflammatory biomarkers and cardiovascular diseases suggesting their utility to identify the risk of an acute ischemic event and the detection of vulnerable plaques. The emerging inflammatory markers are well divided for diagnosis and prognosis and plaque instability of coronary artery disease. Some of them, the lectin-like oxidized low density lipoprotein receptor-1 can be important both in diagnosis and in the evaluation of plaque instability, other are inserted in the above reported classification. The emerging inflammatory markers in acute-phase include amyloid A, fibrinogen and pentraxin 3 while myeloperoxidase, myeloid-related protein 8/14 and pregnancy-associated plasma protein-A are recognize markers of plaque instability. Lastly, some studies demonstrated that circulating miRNAs are involved in coronary artery disease, acute myocardial infarction and heart failure.

Key words: Coronary artery disease; Plaque instability; Inflammation; Acute phase; Biomarkers

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Core tip: In this review we want to focus the reader's attention on the differences between inflammatory markers of cardiovascular risk already accepted by the scientific community and the emerging markers in order to encourage the healthcare services to improve laboratory techniques in early diagnosis and more precise evaluation of the risk. Is also important to use a classification according to the stage where the patient is located regarding emerging inflammatory markers for diagnosis, prognosis and plaque instability.

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INTRODUCTION

Atherosclerosis is largely recognized as a chronic inflam-

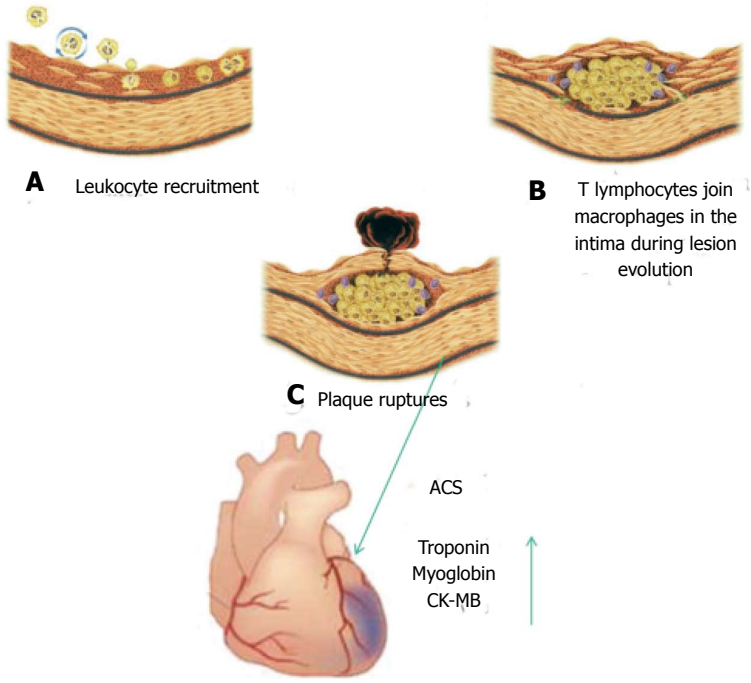

Figure 1 Phases of coronary atherosclerosis. Events implicated in the progression of acute coronary syndrome (ACS).

matory disorder caused by vascular and extravascular factors[1,2] and coronary artery disease (CAD) is its common manifestation. CAD could result in the development of acute coronary syndrome (ACS), which is often associated with breakage of an atherosclerotic plaque and partial or complete thrombosis of the related artery.

In these years a large number of studies permitted a better knowledge of the events implicated in the progression of ACS: here we summarize them (Figure 1). In these processes there is a recruitment of macrophages, that secretes lytic enzymes such as metalloproteinases. The atheroma core is constituted by foam cells and extracellular lipids shrouded by of smooth-muscle cells and collagen matrix. Plaque ruptures release adhesion molecules and soluble factors, such as D-dimers, von Willebrand factor and plasminogen activator inhibitor-1 that have an important role in thrombus formation. In a few hours after thrombus formation, but before the initiation of coronary ischemia, albumin is released. Troponin, myoglobin, and creatine kinase-MB are time-dependent release components associated with myocardial necrosis[3] (Figure 1). The extent of these events influences the circulating troponin level[4]. Therefore it is important to identify the fundamental steps leading to atherosclerotic plaque rupture.

Adequate risk assessment remains the most challenging in individuals classified into low or intermediate risk categories. Inflammation is important in the progression of atherosclerosis and in plaque rupture[1,5]. For this reason, numerous inflammatory markers have been extensively investigated as potential candidates for the enhancement of cardiovascular risk assessment.

Several recent studies have demonstrated the role of inflammation in mediating the stage of CAD, often caused by lipid accumulation.

Moreover the different part of atherogenesis could be related to inflammatory biomarkers that are important for clinical diagnosis, treatment and prognosis of patients with CAD. However, because conventional risk factors do not explain the changes in atherosclerosis, efforts have focused on developing novel biomarkers which identify vulnerable plaques and cardiovascular disease[6,7].

These new laboratory biomarkers should be standardized in variability, sensitivity and specificity from established risk markers. Finally, the cost of the assays has to be acceptable. In this review we analyze the inflammatory markers now considered valid in the stratification of risk for CAD and those emerging, checking if new ones can express something more than the standardized biomarkers.

CONSOLIDATED MARKERS

C-reactive protein

C-reactive protein (CRP), a pentraxin composed of 5 subunits, is an inflammatory marker that may increase in various pathological situations, synthesized mainly in the liver, but it is also produced by leukocytes and adipocytes[8,9].

According considerable evidence, during infection or tissue necrosis circulating CRP may increase 50000 times, but it is also regarded as an independent variable of future cardiovascular events[10].

CRP fosters antigen presentation and phagocytosis attaching to phosphocholine that is usually found in cell membranes and polysaccharides in prokaryotes and fungi and binding to complement C1q complex and factor H[11,12].

Moreover it can attach low density lipoprotein (LDL), and be identify within the plaque[13] where it participates to inflammatory atherogenic processes[14]. CRP is elevated

in patients with acute and chronic coronary syndromes in relation to the composition of the plaque[15,16] and is related to the complications of heart failure[17]. Low plasma levels of CRP indicate a good state of health[18], while increase when the style of life worsens. The MONICA Augsburg Study shows that low quality "Western" diet with low consumption of vegetables, fruit and fiber, extensive use of saturated fat, low physical activity and obesity, are associated with higher CRP levels[19].

Therefore, increment of CRP plasma concentration reflects not good lifestyle choices that lead to a metabolic disequilibrium and inflammation. The study of a large population has revealed that an increase in the levels of CRP (> 3 mg/L, elevated levels) was associated with mortality of 22962 subjects[20].

Ridker *et al*[21] showed that CRP was a better biomarker of cardiovascular diseases than LDL cholesterol. However when measured together, they give better prognostic detail than measured separately[21]. A large prospective study documented a strong association between CRP predictive power and the risks for coronary artery disease[22,23]. Moreover, the Canadian Cardiovascular Society suggested that CRP evaluation in patients at "intermediate risk", could represent a predictive risk of a cardiovascular event from 10% to nearly 20% within the subsequent 10 years[24]. In agreement with this observation, the National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines and the American College of Cardiology Foundation-AHA Task Force on Practice Guidelines affirmed that the evaluation of CRP levels was acceptable for patients at intermediate risk[25,26].

Another study regarding people at intermediate risk for a cardiovascular event showed that the values of CRP and fibrinogen could help to prevent one additional event over a period of 10 years for every 400 to 500 people screened.

Current knowledge, however, suggests that the CRP concentration might reflect the vulnerability of the atheromatous lesion and the prospect of plaque rupture[5,27,28]. The development of high-sensitivity CRP (hs-CRP) assays has been useful to investigate its role in predicting first cardiovascular events.

Cytokines

Interleukin 1 (IL-1), IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor alpha (TNF-α) are the main investigated cytokines among those which predict cardiovascular events involved in vascular inflammation and atherosclerosis[29,30]. IL-1 and IL-6, regulate CRP production *via* direct stimulation of the hepatocytes[31]: IL-6 may increase plaque instability modulating the expression of TNF-alpha, and MCP-1[32]. Elevated IL-6 levels in healthy men correlated with increased risk for future MI independently from hs-CRP[33]. According to some author IL-6 seems to be a marker more sensitive and specific than CRP in vascular inflammation and CRP studies show a weaker association with cardiovascular disease than cytokines[34,35]. In the Fragmin study (FRISC-Ⅱ), IL-6 increment above 5 ng/L was related with a mortality from 6- to 12-mo without a relationship with troponin and hs-CRP[36].

Therefore, IL-6 plasma concentration results as an effective independent index of increased mortality in unstable CAD and characterizes subjects who advantages of an initial invasive strategy. In addition, the intensity of plaque inflammation and its vulnerability seems to be linked with plasma IL-6 levels[36].

Some studies showed that IL-1 could have a regulatory function in the atherosclerotic development suggesting its modulation in vascular smooth muscle cell mitogenesis[37,38], in leukocyte adherence to vascular wall[39,40], in LDL metabolism[41,42], in extracellular matrix proteins[43] and in vascular permeability[44]. Moreover, IL-1 has been found to suppress vascular contractility[45] and induce pro-coagulant activity[46].

Several years ago, increased levels of IL-1α and IL-1β were detected in human atherosclerotic plaque, suggesting their local synthesis[47]. Moreover, IL-1 protein has been detected in macrophages from damaged carotid arteries[48]. In the macrophages IL-1β secretion seems to be induced by the cholesterol crystals present into the plaque[49].

The presence of increased IL-1β plasma concentration in patients affected by unstable angina indicates its important role in the acute stage[50]. However, in mouse models conflicting roles have been reported for IL1β: on the one hand the absence of IL-1β is associated with a reduction of atherosclerotic severity[51], on the other hand, IL-1β inactivation seems to be related to atherosclerotic plaque stability[52].

TNF-α plays a role in myocardial dysfunction and remodeling after acute coronary events[53]. On behalf of this effect, the CARE study showed that TNF-levels increased in recurrent coronary events after a MI compared with controls[54].

The chemokine MCP-1 recruits monocytes into the arterial wall activating these cells to induce endothelial injury[55]. In addition, a positive correlation between MCP-1 levels and the extent of coronary atherosclerosis was found in the coronary circulation of patients with unstable angina[56,57]. Moreover, MCP-1 levels have been found to correlate with older age[58], hypertension[59], hypercholesterolemia[60], and kidney failure[61], while an inverse correlation has been observed with estrogen replacement[62] and HMG-CoA reductase inhibitor therapy[60]. In several studies with small number of subjects, plasma MCP-1 levels were highest among patients with acute coronary syndromes (ACS), intermediate with stable coronary disease, and lowest among healthy control subjects.

IL-10 is an important factor for its anti-atherogenic property. In fact patients with high IL-10 levels had a reduced mortality compared with those that have only elevated CRP[63].

In 158 patients affected by stable CAD, during a 7-year follow-up period, the multivariate analysis of 10 cytokines showed IL-8 as the only independent marker for cardiovascular diseases[64]. In summary, even if the results are still controversial, in our opinion among consolidated

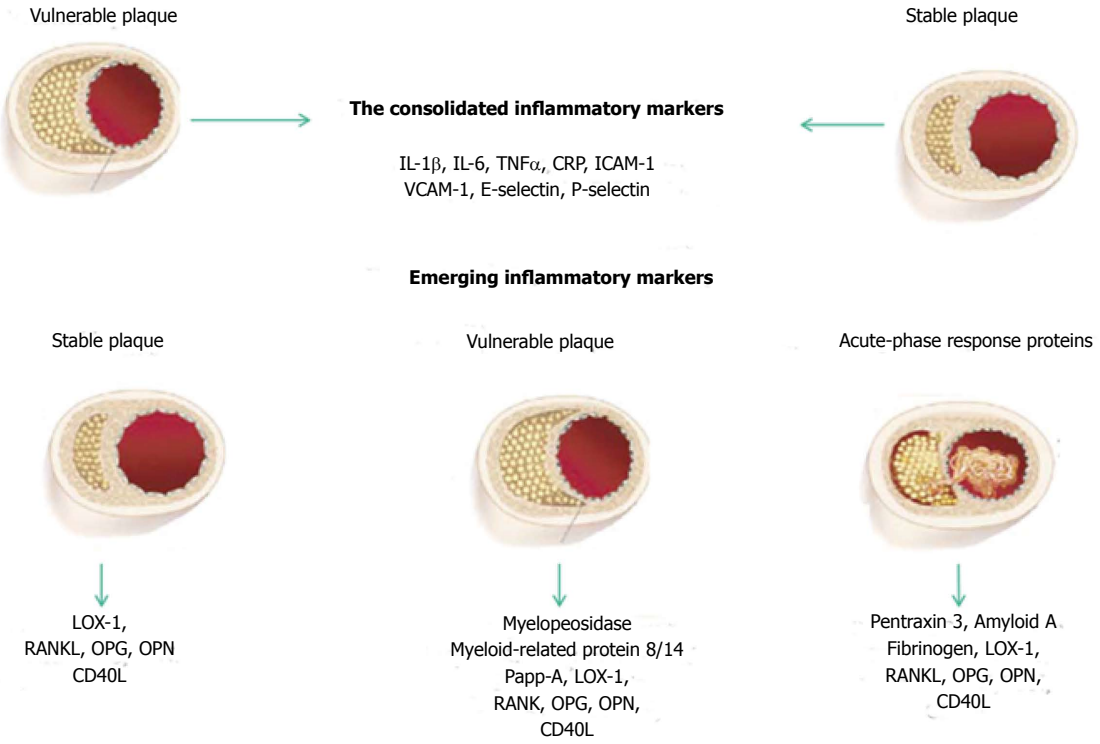

Figure 2 Consolidated and emerging inflammatory markers. A new approach to establish the risk for coronary artery disease. Today new inflammatory markers are studied and classified according to their role in the development of coronary artery disease. TNF-α: Tumor necrosis factor alpha; IL: Interleukin; CRP: C-reactive protein; CAM: Adhesion molecules; OPG: Osteoprotegerin; OPN: Osteopontin. PAPP-A: Pregnancy-associated alpha plasma protein A; LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1.

cytokine, IL-6 represents the best prognostic biomarker in CAD.

Adhesion molecule

Although very broad, adhesion molecules (CAMs) may be regarded as inflammatory markers of cardiovascular risk. Soluble CAMs (ICAM-1, VCAM, P and E selectines) are released from the surface of the cell and reflect cellular activation[65]. CAMs induce the bind between leucocytes, platelets and vascular wall[66]. After the adherence to the endothelium, the leucocytes transmigrate into the arterial wall determining the first phase of atherosclerosis[66].

Several studies reported an association between the increase of plasma CAM concentration and the risk of cardiac events[34,67,68], but their role in CAD prognosis have not been established because their finding are still quite confused.

In patients with stable CAD, CAMs plasma concentrations were measured and informations on cardiovascular events were collected for some years. Among CAMs, only VCAM-1 resulted independently significant with future cardiovascular events[69].

In agreement with this study, other authors observed that the concentrations of sVCAM-1 > 780 ng/mL and CRP > 3 mg/L corresponded to a sensitivity > 90% for predicting future events in patients affected by acutely ACS[70].

On the contrary, other studies did not confirm these findings for sVCAM-1; instead, they suggested that CRP and sICAM-1were useful for identifying the risk of a cardiac event in patients with unstable angina who underwent coronary stenting[71]. Finally, another prospective study showed that only P-selectin and cardiac troponin I, but not the other CAMs, were significantly higher among patients who had a serious cardiac event during the subsequent 3 mo[72].

EMERGING INFLAMMATORY MARERS

The lack of "traditional" risk factors cannot make totally free of the disease and new emerging markers of inflammation have been studied in the effort to identify biomarkers predicting the risk, and at the same time reflecting plaque instability in the early or in the acute phase. On the bases of these studies, we must point out that today is also in use a classification according to the stage where the patient is located (Figure 2).

EMERGING INFLAMMATORY MARERS FOR DIAGNOSIS, PROGNOSIS AND PLAQUE INSTABILITY

Lectin-like oxidized low-density lipoprotein receptor-1

Clinical studies have demonstrated that well-known coronary risk factors, including metabolic diseases, hypertension, obesity and smoking, are associated with oxidative stress. When the LDL are exposed to oxidative stress, they are caught in the vessel and oxidized (ox-LDL). Oxidized LDL promotes the synthesis of a large variety of cytokines and chemokines by the endothelium. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1)

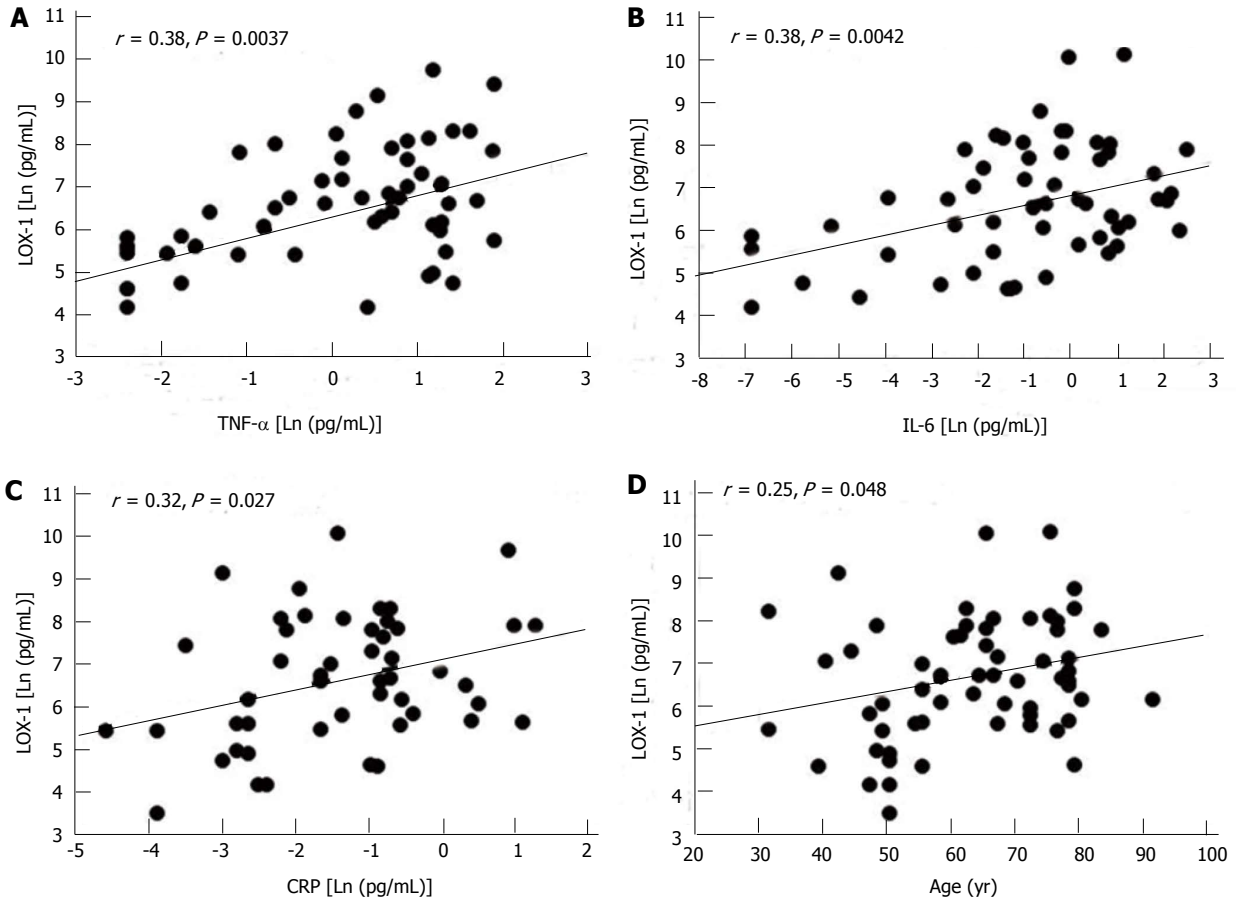

Figure 3 Regression analysis of LOX-1. From Lubrano *et al*[76], 2008. Positive association between circulating levels of LOX-1 and inflammatory markers. TNF-α: Tumor necrosis factor alpha; IL: Interleukin; CRP: C-reactive protein; LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1.

appears to be an important receptor for ox-LDL in endothelial cells[73]. LOX-1 not only allows the passage of oxidized lipids in the cells, but as already described, may cause endothelial dysfunction/apoptosis, inflammation, and the increase smooth muscle cell number favoring the formation of atheroma[74-76].

Moreover LOX-1 increment was observed to be associated with cardiovascular risk factors like hypertension and metabolic disorder. In a population of patients affected by CAD, our previous studies showed a positive relationship between circulating levels of LOX-1 and inflammatory markers: this work suggested also that LOX-1 levels increased with the severity of the disease[76] (Figure 3). Other authors underlined the importance of this novel biochemical marker for the stratification risk of the population and therapeutic strategy for CVD.

Overt cardiovascular disease is typically preceded by a long period of sub-clinical cardiovascular disease and sub-clinical atherosclerosis can be present for decades before the occurrence of a myocardial infarct event. Soluble LOX (sLOX-1) has shown to be informative either early or late in the process disease[77-79].

Recent study observed that the circulating levels of sLOX-1 are very high in acute coronary syndrome and that the plateau value is reached before troponin T, highlighting the instability of the plaque[80].

It has been reported that serum levels of sLOX-1 are also specifically elevated in acute coronary syndrome and the peak value has been reported to rise before troponin T[80]. In conclusion, sLOX-1 levels are related to the prognosis of acute coronary syndrome and reflect the instability of plaque[78].

Nuclear factor-kappa B, osteoprotegerin, osteocalcin, osteopontin, CD40

Receptor activator of nuclear factor Kappa-B ligand (RANKL) is the ligand of the receptor inducer factor-κB (NF-κB) and belongs to the family of cytokines TNF-related. It is synthesized by T cells and stromal/osteoblastic cells and is a strong chemotactic factor for human monocytes[81]. RANKL-stimulated microvascular endothelial cells favor monocyte adhesion and trans endothelial migration thus increasing the recruitment of osteoclast- and osteoblast like cell precursor[81,82].

Osteoprotegerin (OPG) synthesized in osteoblasts is part of the TNF super-family. It binds to RANKL thereby preventing interaction with its transmembrane receptor[83].

RANKL and OPG have been shown to be potentially valuable markers for a better assessment of coronary calcification and cardiovascular risk associated with it. It has been observed that RANKL and OPG may play a key role in maturation and calcification of atherosclerotic

plaque[84,85]. In fact these factors increased in serum of post-infarction of atherosclerotic animal models and of humans with unstable angina[86,87].

Osteocalcin, a protein found in bone and dentin and also synthetized in mononuclear cells, has been related with the severity of aortic calcification[88].

Osteopontin (OPN), an extracellular matrix protein and pro inflammatory cytokine, facilitates the recruitment of monocytes/macrophages through its adhesive domain[89] and promotes the inhibition of vascular calcification In fact it is increased in patients with vascular calcification resulting more like a marker than a mediator of atherosclerosis progression[90].

CD40, a member of TNF family, is also a stimulatory receptor on antigen-presenting cells of the immune system that induces inflammatory processes through the binding of the CD40 ligand (CD40L). Elevated levels of soluble CD40L (sCD40L) have been found in patients with hypercholesterolemia, ACS and cardiovascular disease. sCD40L is also associated with atherosclerosis and plaque instability[91]. In the CAPTURE trial, increased sCD40L (> 5 g/L) was related to a 6-mo mortality or nonfatal MI suggesting that sCD40L may be an independent risk marker of cardiovascular events. Statins, antihypertensive drugs, and antiplatelet agents have been shown to modulate it[92]. Moreover, sCD40L was found to be increased in smokers and positively associated with both total cholesterol and biomarkers of inflammation. However, it was not reported as an independent biomarker for the risk of MI[93].

ACUTE-PHASE RESPONSE PROTEIN

Quantitative and qualitative changes of inflammatory markers are able to identify the acute stage of the disease. The plaque ruptures cause the consequent platelet aggregation and subsequent thrombosis, the final stage in which atherosclerosis leads to acute ischemic syndromes of AMI and sudden death[5].

The literature well documented the association between serum concentrations of acute phase proteins and the onset of coronary heart disease and myocardial infarction[94,95]. The emerging inflammatory markers in the acute-phase include pentraxin 3 (PTX3), amyloid A, and fibrinogen[96-102].

Pentraxin-3

Pentraxins are a superfamily of soluble proteins with cyclic multimeric structure[103]. Among these, PTX3 a protein characterized by a long N-terminal domain, results as an important player in immunity and inflammation[104]. Dendritic cells, macrophages and endothelial cells produce PTX3 in response to IL-1 and TNF[105]. Moreover increased plasma PTX3 levels were observed in patients with cardiovascular disease and resulted also more closely related than CRP levels in acute phase of cardiac damages[106] suggesting that it could be a sensitive and specific prognostic indicator[107].

It is been also hypotheses an association of PTX3 in individuals with stable coronary artery disease and kidney dysfunction. However, an adjustment for the estimated glomerular filtration rate modestly attenuated these associations[108]. By immune histochemical staining PTX3 was strong expressed on the surface of lumen and within the atherosclerotic plaque in humans and animal models[96,97,109]. Moreover, in the same experimental models, soluble PTX3 increased in the early phase after ischemic heart events and PTX3 mRNA and protein expression enhanced in the ischemic area of the heart[110].

In a prospective study of patients with myocardial infarction and ST elevation, PTX3 predicted 3-mo mortality while other markers such as the liver-derived short pentraxin CRP or NT-proBNP, TnT, CK did not[107]. In patients with unstable angina pectoris within the six hours of the chest pain, PTX3 resulted to be more specific for ACS than neutrophil activating peptide-2 and cardiac troponin I (cTnI)[111,112].

Amyloid A

Serum amyloid A (SAA) proteins are a family of apolipoproteins associated with high-density lipoprotein (HDL) and are now considered emerging markers of inflammation. In fact elevated SAA levels are present in coronary artery disease and indicate worse prognosis in CAD. Therefore actions involved to reduce SAA levels could improve the conditions of patients with acute CAD[113].

A study of Kosuge *et al*[99] reported that, in patients with ACS, increased SAA levels were associated with cardiovascular events within 30 d, without any relationship with CRP level. Therefore these data indicated SAA more useful predictor than CRP in these patients.

In the high serum SAA group the left ventricular ejection fraction, measured during follow-up, was significantly lower than in the low serum SAA group and more frequent complications, such as cardiac rupture, carcinogenic shock, subacute thrombosis, and cardiac death, were also present[100].

Furthermore SAA levels were quite well associated with coronary artery disease with a predictive risk for cardiovascular events within 3 years, while this did not happen with hs-CRP[114]. In a substudy of TIMI 11A, elevated SAA levels predicted increased risk of 14-d mortality in patients with ACS[115]. In a Women Ischemia Syndrome Evaluation study, in which women were referred for coronary angiography because of suspected ischemia, elevated SAA values were correlated with angiographic severity of CAD and 3-year risk for cardiovascular events[114]. At the same time, no relationship was observed between SAA levels and recurrent Coronary Events[116].

Fibrinogen

Several studies have indicated fibrinogen as a predictive marker in CAD[117]. Fibrinogen is involved in platelet aggregation, endothelial injury, plasma viscosity and play a central role in the formation of thrombus.

Epidemiological data have shown the important predictive role of fibrinogen in CAD, identifying it as an emerging risk factor because its measurement may improve the estimation of absolute risk obtained by conventional

risk factor for CV[117].

Although it is still discussed the role of fibrinogen as inflammatory markers of risk, many studies indicated an association of hyperfibrinogenemia with atherotrhombosis.

Already in the past, some authors have demonstrated that the risk estimation for CAD could be double when fibrogenemia was also evaluated[118].

Emerging Risk Factors Collaboration showed that, the measurement of fibrinogen level in patients at risk for CAD, could prevent an additional event in the next 10 years for every 400-500 people studied[119].

However, also recent results show that the evaluation of fibrinogen during MI may be useful in identifying patients at high risks for future acute events[102].

BIOMARKERS OF PLAQUE INSTABILITY

The main cause of the acute myocardial infarction (AMI) is the plaque rupture, so that it is important to investigate new markers for early diagnosis of plaque instability.

Due to its sensitivity and specificity, troponin is commonly used in the diagnosis of ACS, even if it provides only indirect details on myocardial necrosis induced by embolization of atherothrombotic material, late event of ACS.

Inflammation is a process that is intensified in plaque instability, so that the markers of inflammation may provide indications of cellular processes related to its formation before it occurs myocardial necrosis[120].

Myeloperoxidase

Myeloperoxidase (MPO) is an enzyme produced by leukocytes that induces the formation of oxygen free radicals and is considered to be one major contributor in the formation and rupture of the plaque[121].

In patients with ACS, MPO produced by neutrophils, is considered a marker of plaque vulnerability as noted by several studies[122, 123].

Yunoki *et al*[124], 2013 observed that the plasma levels of MPO have a significant inverse correlation with levels of paraoxonase-1 bound to HDL, especially, in patients with stable and unstable angina pectoris, suggesting that a mismatch between pro oxidants and anti-oxidants may contribute to the progression of coronary plaque instability[124].

Myeloid-related protein 8/14

Myeloid-related protein 8/14 (MRP8/14), is a heterodimer consisting of two proteins that bind calcium, calgranulin A and B, which play an important role in the signaling pathways of calcium, in cell cycle progression, cell differentiation, and in the interaction between the cytoskeleton and membrane[125]. MRP-8/14, also called calprotectin, is synthesized by activated monocytes and neutrophils, and is a pro-inflammatory protein expressed in atherosclerotic plaques.

High concentrations of MRP8/14 in the systemic circulation may reveal the presence of plaques before necrosis markers suggesting it as a good candidate for the management of ACS unstable.

PAPP-A

PAPP-A is a high-molecular-weight zinc-binding metalloproteinase. PAPP-A was independently associated with recurrent cardiovascular events in patients with ACS. This finding supported the potential usefulness of PAPP-A as a biomarker in patients with ACS[126]. Moreover as described by Mahto *et al*[127] PAPP-A is the reliable marker which can discriminate the cases of MI from unstable angina and controls[127]. Another study has suggested PAPP-A to be a predictor of mortality or myocardial infarction in patients with ACS[128].

Role of microRNAs in CAD

MicroRNAs (miRNAs) are short non-coding RNA molecules that regulate gene expression post-transcriptionally through suppression or degradation of target messenger RNA (mRNA).

MiRNAs were found in the circulating blood and are differently induced in patients with CAD, AMI, and heart failure[129-132].

Of interest is miR-155, which proved to be a new component of inflammatory signal transduction pathways in the pathogenesis of atherosclerosis. In fact the expression of miR-155 is considered to be a prospective marker for predicting the prognosis of CAD since it is found to be expressed mainly in patients with CAD compared to healthy subjects[133].

CONCLUSION

Inflammatory biomarkers appear to have an important prognostic value in patients with cardiovascular disease and may be useful in the diagnosis of apparently healthy subjects without known CAD who cannot be assessed with conventional risk factors.

Inflammatory biomarkers may have prognostic value for future cardiovascular risk among those at high risk or with documented cardiovascular disease. They also may be useful for identifying apparently healthy individuals, without known CAD, who may be at a higher risk than estimated by traditional risk factors.

Although recent data demonstrate that there is a close association between inflammatory biomarkers and coronary artery disease, further studies must be carried out taking into account also some important criteria typically used in the selection of a new biomarker: discrimination, calibration and reclassification, i.e. the ability of a test to discern between those that will face the disease from those that will be free, the assessment of the risk factor predicted and observed, classification in categories of low, intermediate and high risk for CAD[134].

In our opinion the best candidate for this role is LOX-1; it was observed to be associated with cardiovascular risk factors like hypertension and metabolic disorder, showing its positive relationship with inflammatory markers and

its increment with the severity of the disease[76] (Figure 3). Moreover it was elevated in acute coronary syndrome and the peak value has been reported to rise before troponin T reflecting the instability of plaque[80].

In conclusion, the findings observed in a decade showed that LOX-1 could represent an important marker for clinical characterization of coronary artery disease and a target for new drugs to reduce its expression and production.

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Ledipasvir and sofosbuvir: Interferon free therapy for hepatitis C virus genotype 1 infection

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Abstract

Hepatitis C virus (HCV) has infected more than 200 million people around the globe. From 2001-2011, interferon plus ribavirin remained the standard of care for patients with HCV infection. The therapy had a limited response with a number of side effects. Recently, results for phase III trials of ledipasvir and sofosbuvir combination therapy have been announced. In treatment naïve patients, 12 wk of therapy with ledipasvir and sofosbuvir showed a sustained virological response (SVR) rate of 99%. In treatment experienced patients, 12-24 wk of therapy with ledipasvir and sofosbuvir in the absence or presence of ribavirin showed an SVR rate of 94%-99%. In cirrhotic patients the rate of SVR was 86% and 99% for 12 and 24 wk of therapy, respectively. The ledipasvir and sofosbuvir therapy showed very good results in different subgroups of patients regardless of patient's race, alanine aminotransferase levels, sex and host genetic factors. The combination therapy was well tolerated with no emergence of resistant mutants. The most common adverse effects were nausea, headache and fatigue. With the availability of interferon free therapy with minimal adverse effects, it will be easy to decrease the future morbidity and mortality caused by HCV infection.

Key words: Hepatitis C; Interferon; Ledipasvir; Sofosbuvir; Genotype

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Core tip: The interferon based therapy for hepatitis C patients has a limited response with a number of adverse effects. The ledipasvir and sofosbuvir combination therapy showed a sustained virological response (SVR) rate of 99% in treatment naïve patients. The rate of SVR was 94%-99% in treatment experienced patients, while in cirrhotic patients the rate of SVR was 86%-99%. The treatment response was not affected by ethnicity or host genetic factors.

Waheed Y. Ledipasvir and sofosbuvir: Interferon free therapy for hepatitis C virus genotype 1 infection. *World J Virol* 2015; 4(1): 33-35 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i1/33.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i1.33

TO THE EDITOR

Hepatitis C virus (HCV) infection is a major health problem around the globe, with more than 200 million people infected worldwide. Although the rate of HCV infection is continuously declining, the rates of HCV associated morbidity and mortality are continuously increasing.

From 2001-2011, interferon and ribavirin therapy remained the standard of care for patients living with HCV. The therapy had a limited response with a number of side effects. The major adverse effects associated with interferon administration were flu like symptoms, cytopenia and depression, whereas ribavirin therapy causes fatigue, anemia, rash and pruritus. The major objective of recent treatment regimens is to eliminate the interferon and ribavirin from the treatment regimen so that the adverse effects of therapy can be reduced and the therapy become available for patients who are ineligible for the interferon and ribavirin therapy.

Sofosbuvir is a nucleoside analogue that can inhibit the HCV polymerase, approved by the Food and Drug Administration for the treatment of patients living with HCV. Ledipasvir is an inhibitor of HCV NS5A protein, showing antiviral activity against HCV genotype 1 infection.

In a phase Ⅱ clinical trial, 100 patients with HCV genotype 1 infection who were treatment naïve or previously treated with protease inhibitors were enrolled at a centre in the United States. The patients were given a fixed-dose combination of sofosbuvir (400 mg) and ledipasvir (90 mg). In cohort A, 60 treatment naïve, non-cirrhotic patients who were given sofosbuvir plus ledipasvir (8 wk), sofosbuvir plus ledipasvir along with ribavirin (8 wk), or sofosbuvir plus ledipasvir (12 wk) showed an SVR rate of 95%, 100%, and 95% respectively. In cohort B, 40 previous non-responders to protease therapy were included. They were given sofosbuvir plus ledipasvir (12 wk) or sofosbuvir plus ledispavir along with ribavirin (12 wk), and the sustained virological response (SVR) rate was 95% and 100%, respectively[1]. The sofosbuvir-ledipasvir combination therapy cured most of patients with HCV genotype 1 infection, irrespective of their treatment history. Further investigations were required to optimize the treatment duration and the role of ribavirin in treatment response.

In a phase Ⅲ clinical trial, 865 previously untreated patients were enrolled and they were randomly divided into four groups. Group 1 received ledipasvir and sofosbuvir for 12 wk and showed an SVR rate of 99%. Group 2 received ledipasvir and sofosbuvir along with ribavirin for 12 wk and showed an SVR rate of 97%. Group 3 received ledipasvir and sofosbuvir for 24 wk and showed an SVR rate of 98%. Group 4 received ledipasvir and sofosbuvir along with ribavirin for 24 wk and showed an SVR rate of 99%. The study concluded that the 12 wk therapy with ledipasvir and sofosbuvir was highly effective for patients living with HCV genotype 1 infection. No additional benefit was observed by the addition of ribavirin or by the extension of therapy to 24 wk[2].

In another phase Ⅲ trial, 440 previously treated patients were enrolled, 20% of whom had cirrhosis. The patients were given ledipasvir and sofosbuvir in the presence or absence of ribavirin from 12 or 24 wk. The rate of SVR achieved was 94%-99%. In patients with cirrhosis the rate of SVR was 86% (ledipasvir-sofosbuvir) and 82% (ledipasvir-sofosbuvir plus ribavirin) with 12 wk of treatment, while the rate of SVR was 99% (with both regimens) in patients having 24 wk of treatment. The study concluded that the single tablet of ledipasvir–sofosbuvir showed a better rate of SVR even in the patients who were not responders to the interferon based therapy[3].

The ledipasvir and sofosbuvir therapy produced very good results in different subgroups of patients regardless of patient's race, alanine aminotransferase levels, sex and host genetic factors. The combination therapy was well tolerated. No S282T variant was observed. The most common adverse effects were nausea, headache and fatigue[2-4].

A total of 1952 patients were enrolled in three different phase III trials of ledipasvir and sofosbuvir, out of which 97% showed SVR[2-4]. Out of the remaining 3%, half of them withdrew consent or were lost to follow-up. Undetectable viral RNA was not achieved in only two patients. The rate of relapse was observed in only 2% after stopping therapy. The rate of relapse was also linked with the treatment duration. The rate of relapse was observed in 5%, 2% and 0.2% of patients who received 8 wk, 12 wk and 24 wk of treatment, respectively[5].

With the availability of oral, short duration, interferon free therapy with minimal adverse effects, the future morbidity and mortality associated with HCV infection will decrease. The major problem with the therapy is its cost. The cost of 12 wk therapy with sofosbuvir alone is $84000 and the addition of ledispavir will further increase the cost[5]. The high cost of the therapy will affect the goal of providing safe and effective treatment for millions of patients living with HCV around the globe.

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REVIEW

Endothelium-derived essential signals involved in pancreas organogenesis

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Abstract

Endothelial cells (ECs) are essential for pancreas differentiation, endocrine specification, and endocrine function. They are also involved in the physiopathology of type 1 and type 2 diabetes. During embryogenesis, aortic ECs provide specific factors that maintain the expression of key genes for pancreas development such as pancreatic and duodenal homeobox-1. Other unknown factors are also important for pancreatic endocrine specification and formation of insulin-producing beta cells. Endocrine precursors proliferate interspersed with ductal cells and exocrine precursors and, at some point of development, these endocrine precursors migrate to pancreatic mesenchyme and start forming the islets of Langerhans. By the end of the gestation and close to birth, these islets contain immature beta cells with the capacity to express vascular endothelial growth factor and therefore to recruit ECs from the surrounding microenvironment. ECs in turn produce factors that are essential to maintain insulin secretion in pancreatic beta cells. Once assembled, a cross talk between endocrine cells and ECs maintain the integrity of islets toward an adequate function during the whole life of the adult individual. This review will focus in the EC role in the differentiation and maturation of pancreatic beta cells during embryogenesis as well as the current knowledge about the involvement of endothelium to derive pancreatic beta cells *in vitro* from mouse or human pluripotent stem cells.

Key words: Endothelium; Endothelium-derived signals; Differentiation; Pancreas development; Organogenesis

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Core tip: Many studies have demonstrated that endothelial cells (ECs) have an important role in organogenesis. For instance, during embryogenesis, aortic ECs provide specific factors that maintain the expression of key genes for pancreas development. Other unknown factors are also important for pancreatic endocrine specification and formation of insulin-producing beta cells. In addition, by the end of the gestation and close to birth, pancreatic

islets contain immature beta cells with the capacity to express factors that recruit ECs from the surrounding microenvironment and form a functional unit that will lasts for the whole life of the individual. In the present review, we will analyze the current endothelial-derived factors called angiocrine factors that are essential in organogenesis and we will focus the role of these factors in pancreas development and pancreatic beta cells.

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INTRODUCTION

At present, insulin producing cells have been derived from different sources[1-3]. With the emergence of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), that can be plated *in vitro*, the potential production of several cell types including pancreas can be now achieved[2,4-11]. Endothelial cells (ECs) are present at early stages during embryogenesis[12-15]. These cells are the main component of most niches[16]. Therefore, ECs interact early with developing tissues during organogenesis even before they are able to form blood vessels and nourish specific regions[16-19]. It has been reported that the absence of ECs results in agenesis of some organs such as pancreas[20,21]. This fact points out the essential role of EC signaling during organogenesis. Apparently ECs are not only involved in pancreas differentiation, they are essential for endocrine differentiation as well[20]. Endocrine progenitors give rise to immature beta cells that recruit ECs after expression of vascular endothelial cell growth factor (VEGF)[18,20]. These ECs in turn provide factors to promote beta-cell maturation and stabilize β-cell function. In this review, we will be focusing in the role of ECs in the differentiation and maturation of beta cells *in vivo* and *in vitro* with the emerging technology of human PSCs that can be expanded *in vitro*.

ENDOTHELIUM AS AN ENDOCRINE ORGAN

Extensive studies with ECs have demonstrated that these cells play essential roles in immunity, inflammation, angiogenesis, and tumor metastasis. ECs do not only line the interior surface of blood vessels[22,23]. ECs are found at the interface between blood and other cell types. They not only maintain the blood fluid but also have a great plasticity that allows these cells to accomplish several essential functions to maintain homeostasis[18]. However, recent studies demonstrated that the endothelium is capable of releasing growth factors and cytokines that play an essential role in organogenesis[24] (Table 1). The term angiocrine has been proposed by Butler and collaborators to indicate the capacity of ECs to release growth factors and cytokines that may be involved in organogenesis[25]. For instance, bone marrow sinusoidal ECs (SECs) promote differentiation of hematopoietic stem cells (HSCs) through "angiocrine factors" such as hepatocyte growth factor (HGF), Wnt2, and Notch[25,26]. Additionally, Butler and colleagues reported that ECs promote self-renewal of hematopoietic stem cells when these cells are interacting in co-culture[25]. Apparently, Notch ligands expressed by ECs are associated with this response[25]. Another studies indicate that ECs from liver, called sinusoidal ECs (LSECs), express factors such as VE-cadherin, Factor VIII and vascular endothelial growth factors 2 and 3 (VEGF2, VEGF3) and these cells release angiocrine factors that may be involved in liver regeneration[26]. In another work it has been described that angiocrine factors are able to regulate tumor growth[27]. Other studies demonstrated that mice deficient in FLK-1 (VEGF receptor), die at embryonic day E9.5 or E10.5 because immaturity of blood cells and blood vessels[28]. Absence of embryonic liver budding is also present in these mice indicating that ECs play an important role during the early phases of organogenesis. In a similar work, it has been observed that ECs are also involved during the early stages of pancreas development[20]. Another research work indicated that signals from myocardium, such as those exerted by bone morphogenetic protein-2 (BMP-2), can promote epithelial-mesenchymal transformation mediated by ECs[24,29]. In the kidney, VEGF, bFGF, and PDGF coordinate cellular differentiation, proliferation, and migration[30]. It has been suggested that ECs promote the differentiation of endoderm cells toward liver or pancreas through secretion of HGF[17,19,24,28,31,32] (Table 1). At the same time, reciprocal interactions between tissue-specific cell types and ECs ensure coordinated growth and adequate tissue function. For instance, it is known that neurogenesis takes place close to blood vessels in adult brains[33]. Additionally, brain-derived neurotrophic factor (BDNF) secreted by ECs promotes neurogenesis and angiogenesis in the brain of song birds[34]. Another report indicates that pigment epithelium-derived factor (PEDF) is secreted by ECs and enhances self-renewal of neural stem cells (NSCs)[35]. Regarding the pancreas, it has been shown that pancreatic endoderm attract endothelial progenitor cells (EPCs) or angioblasts by expression of SDF-1/CXCL12[36]. Expression of pancreatic and duodenal homeobox 1 (PDX-1) appeared in endoderm cells in contact with angioblasts *via* LIM domain only 2 (LMO2) suggesting that angioblasts may induce expression of PDX-1[37]. Functional blood vessels may induce differentiation before they carry blood. However, some blood factors such as sphingosine-1-phosphate (SIP) are important for differentiation and maturation[38]. For instance, it has also been described that beta-cell differentiation can be regulated by oxygen tension *via* hypoxia-inducible factor 1 alpha (HIF-1α)[39]. This fact suggests that blood factors can also be involved in the

Table 1 EC-derived factors related to organogenesis

Angiocrine factors	Source	Target	Ref
Notch ligands (1 and 2)	Bone marrow ECs; brain ECs	Neural progenitors and HSCs	[40,41]
VEGF	ECs	ECs and several tissues such as islets	[12,42,43]
bFGF	ECs	ECs and several tissues such as islets	[43]
PDGF	Brain capillaries	ECs	[42]
HGF	Lunga capillaries, SECs, islet capillaries	Lung epithelium, hepatocytes, islet beta cells	[26,31,44,45]
Endothelins	ECs	Lung, neural cells	[46]
EG-VEGF	ECs	Endocrine glands	[32]
Brain-derived neurotrophic factor	Brain microvascular endothelium	Neuronal precursors, islet endothelium	[33]
Pigment epithelium-derived factor	Brain capillaries	Neural stem cells	[35,47]
Vessel-derived stromal-derived factor 1 (SDF-1/CXCL12)	Microvascular endothelium	Endoderm and pancreatic beta cells	[48]
Wnt2	Sinusoidal ECs	Hepatocytes	[26]
S1P	Plasma (plateles)	Pancreatic multipotent progenitor cells	[38]
CTGF	Pancreas capillaries	Pancreatic endocrine cells	[49]
Laminin	Islet capillaries	Islet endocrine cells	[50]
Collagen IV	Islet capillaries	Islet beta-cells	[51]
BMP-2	ECs/MSCs	Islet beta-cells	[52,53]
BMP-4	ECs/MSCs	Islet beta-cells; hepatocytes; cardiomyocytes	[52,53]
BMPR1A	ECs/MSCs	Islet beta-cells; hepatocytes; cardiomyocytes	[52,53]

ECs: Endothelial cells; BMP: Bone morphogenetic protein; VEGF: Vascular endothelial cell growth factor; HGF: Hepatocyte growth factor; bFGF: Basic fibroblast growth factor; PDGF: Platelet-derived growth factor; CTGF: Connective tissue growth factor.

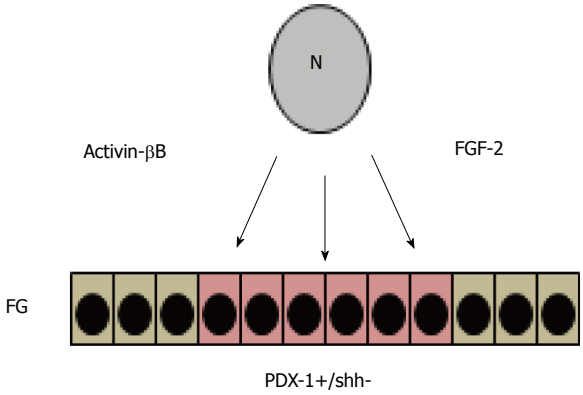

Figure 1 Early cell-cell interactions that give rise to pancreatic cells derived from definitive endoderm of the foregut (FG). Factors released by notochord (N) such as activin-βB and FGF-2 permit expression of PDX-1 and suppress expression of sonic hedgehog. FGF: Fibroblast growth factor; PDX: Pancreatic and duodenal homeobox.

complete differentiation and maturation of pancreatic endocrine cells.

ENDOTHELIUM ROLE IN THE PANCREATIC NICHE

The pancreas originates from ventral and dorsal buds formed in the foregut at 8.5 d post-coitum (d.p.c) of gestation in mice and Carnegie stage 12 (CS12) in humans[54-56]. The cells that composed these buds express transcription factors such as PDX-1 which is a key regulator of pancreas development[57-59]. However, before these cells express these genes, the cells interact with other surrounding cells such as those that compose the notochord and factors such as fibroblast growth factor-2 (FGF-2) and activin-βB suppress the expression of sonic hedgehog (*SHH*) locally and promotes expression of PDX-1 in the subjacent endoderm[60-63] (Figure 1).

Once the mesoderm layer starts proliferating, other signals from aortic endothelial cells (AECs) and mesenchyme (MCs) continue interacting with these PDX-1 expressing cells that give rise to acinar cells that harbor pancreatic exocrine and endocrine progenitors[64] (Figure 2). As these progenitors continue receiving more surrounding instructive signals, definition of cell function is established and the exocrine cells form acini while the endocrine cells form islets of Langerhans[54] (Figure 3). However, these islets contain immature beta cells that will become more mature after islet vascularization that allows closer interaction between beta cells and ECs.

Embryonic endothelium and endoderm pre-patterning

The vascular system is one of the first tissues that develop during embryogenesis. Mesodermal progenitors coalesce in the yolk sac and give rise to endothelium and blood cells[15]. Endothelial cells exert inductive effects on specific points were they are in contact with pre-patterned definitive endoderm (DE) cells of the FG[20]. DE forms during gastrulation and Nodal, a member of transforming growth factor β (TGFβ) family, plays a central role in DE formation[65]. At these points of effective cell-cell interactions, the gut endoderm has to be competent to respond to EC-derived signals. Competence of these cells takes place during gastrulation when the mesoderm germ layer invades the middle area between primitive ectoderm and endoderm[66].

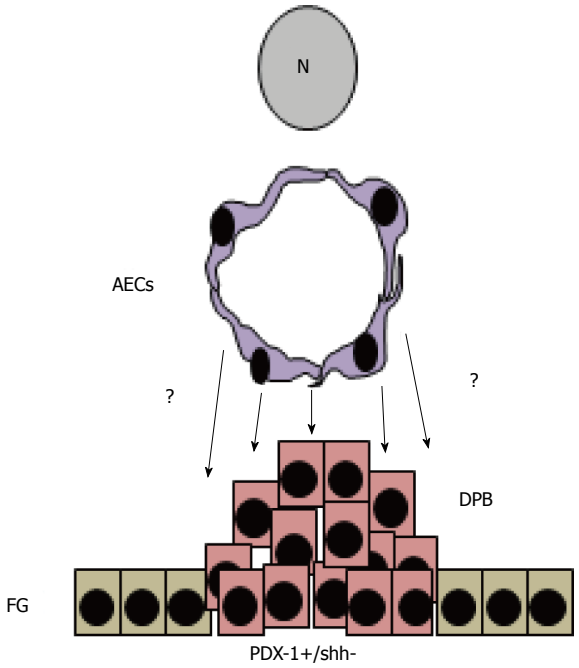

Figure 2 Endothelial-derived signals maintain expression of PDX-1 and promote pancreatic endocrine differentiation. Once the aorta is forming, aortic ECs (AECs) interact closely with FG/DE cells that maintain expression of PDX-1 and form the dorsal pancreatic bud (DPB). ECs: Endothelial cells; DE: Definitive endoderm; FG: Foregut; PDX: Pancreatic and duodenal homeobox.

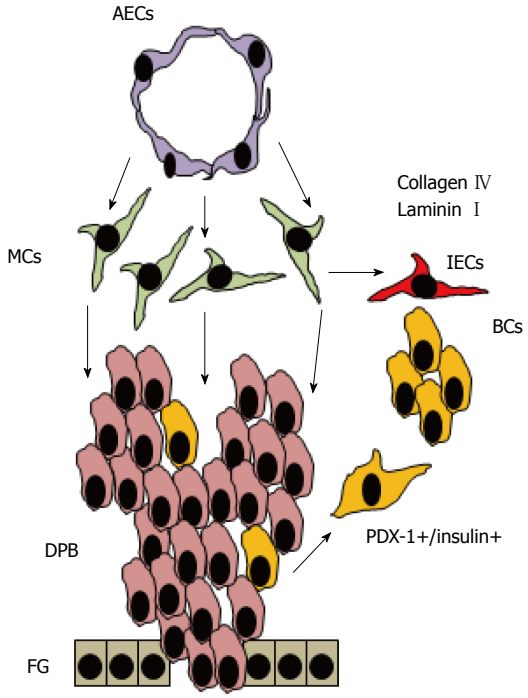

Figure 3 Endothelial-derived signals promote the survival of pancreatic mesenchyme which is essential for pancreas development. Mesenchymal cells (MCs) appear between AECs and the dorsal pancreatic bud (DPB) and promote proliferation and survival of differentiated cells. Immature beta cells (BCs) that co-express PDX-1 and insulin migrate toward the mesenchyme and form cell clusters that will become islet of Langerhans that will recruit ECs that become islet ECs (iECs) and produce collagen IV and laminins which promote insulin expression. AECs crosstalk with MCs and maintain the integrity of these cells toward adequate exocrine and endocrine pancreas development. AECs: Aortic ECs; ECs: Endothelial cells; PDX: Pancreatic and duodenal homeobox.

Apparently mesoderm-derived cells are required to maintain the phenotype of posterior endoderm that includes the site where pancreas and duodenum will be formed. Therefore, anterior-posterior (A-P) endoderm axis will be sustained by the presence of mesoderm-derived factors such as Wingless-type MMTV integration site family (Wnt), fibroblast growth factor (FGF), bone morphogenetic proteins (BMPs), and retinoic acid (RA)[63,67]. For instance, high signaling of the canonical WNT/β-catenin pathway promotes endoderm posterior pattern with foregut-derived structures such as pancreas and liver[68]. Interestingly it has been found that some of these factors are produced by ECs[13,42,53,69-71].

Pancreatic specification induced by surrounding endothelium

Endothelial signaling is required to induce insulin gene expression during pancreas development[20]. Cell-cell interactions between definitive endoderm and aortic endothelial cells take place at about 9-10 d.p.c. in mice and give rise to PDX-1 expressing cells[18,20]. Apparently, these interactions are also essential to promote insulin expression in pre-patterned endoderm[20]. However, signals from the developing pancreas to embryonic endothelium also promote endothelium-specific phenotype and these interactions are crucial for adequate organ function in adulthood[32]. As mentioned above, the first signals to promote expression of PDX-1 come from the notochord that produce factors such as activin-βB and FGF-2[60]. These cross-talk take place between cells from notochord and cells from the subjacent endoderm leading to inhibition of factors such as *SHH* and promotion of PDX-1 expression[60]. However, these permissive signals are apparently replaced by instructive signals from the growing mesoderm and AECs that become in close proximity with pre-patterned endoderm. Interestingly, ECs not only exert these signals directly to the pre-patterned endoderm but also promote the survival of adjacent mesodermal cells that produced essential factors such as Islet-1 (Isl1)[64]. Homozygous mice lacking expression of Flk-1 (*flk-1*$^{-/-}$) had absence of aorta with no formation of dorsal mesenchyme that led to diminished expression of PDX-1 in the subjacent endoderm[64]. However, lateral and ventral mesenchyme were not affected and PDX-1 positive cells also appeared in the ventral endoderm[64]. These findings indicate that aortic EC signaling is essential to maintain the dorsal mesenchyme and therefore to direct differentiation of dorsal pancreatic endoderm. Additionally, at later embryo stage, *flk-1*$^{-/-}$ mice showed dorsal mesenchyme that does not express Isl-1 in the absence of aortic ECs suggesting that endothelial-cell signaling promotes Isl-1 cell expression from dorsal mesenchymal cells[64]. In addition, it has been reported that mesenchymal cells also express bone morphogenetic proteins (BMPs) and that these proteins have a pivotal role in pancreas development[71,72].

Signals from pancreatic mesenchyme toward exocrine pancreas

It is well known that once the mesenchymal cells proliferate between aortic ECs and the foregut endoderm, the aorta is pulled out from the subjacent gut (compare Figure 2 with Figure 3)[20,55]. This fact implies that the subjacent foregut starts receiving signals from the mesenchyme and that a gradient can be formed with diluted signals from aortic ECs. However, it has been demonstrated that ECs maintain the integrity of the subjacent mesenchyme and that absence of aorta promotes apoptosis of the mesenchymal cells and avoids the formation of the dorsal pancreas[64]. As mentioned before, mesenchymal cells between aorta and foregut maintain the expression of PDX-1 in pancreatic endoderm cells through the expression of the transcription factor Isl-1[54]. In addition, signals from mesenchyme such as Fgf-10 are essential to promote proliferation of pancreatic buds that already received signals from ECs[64]. Apparently, the specification of pancreatic fate is determined by permissive signals from notochord and instructive signals by ECs that are maintained by mesenchyme after the aorta is pulled out from the gut. It has been suggested recently that specification of endocrine phenotype also takes place during the close interaction between aortic ECs and pancreatic progenitors that express PDX-1 within the pancreatic bud[54]. Previous experiments indicated that insulin is expressing in foregut explants only after co-culturing with aortic endothelial cells[20]. However, the signals from ECs that promote such specification in the foregut endoderm toward the formation of pancreatic endocrine progenitors are still under investigation. In the same way more characterization is required to identify the signals from ECs that promote survival and adequate function of mesenchyme. Therefore, functional mesenchyme is crucial for appropriate signaling to the subjacent foregut endoderm toward maintenance of the pancreatic phenotype and branching[43].

ENDOTHELIUM ROLE IN PANCREATIC BETA-CELL MATURATION

Pancreatic endothelium during islet neogenesis

Pancreatic endocrine progenitors receive signals from aortic ECs and mesenchyme that determined their fate. However, they remain interspersed with the ductal cells that form the epithelium of the growing branches. At a certain time of development, these cells receive still unknown instructions to migrate from the ductal area to the mesenchyme region. Once in the mesenchyme, these pancreatic progenitors that are apart from ductal cells form the islets of Langerhans[73]. This fact raises two questions: (1) Are there signals from ECs that promote islet neogenesis? and (2) Once the blood vessels are formed are there factors in the blood stream that promote the final maturation of beta cells? The answer to these questions is still unknown. The cells that migrate are pancreatic endocrine progenitors that give rise islets of Langerhans composed of alpha, beta, delta, and PP cells that will produce glucagon, insulin, somatostatin, and pancreatic polypeptide respectively. These islets will be distributed differently into the pancreas and apparently will be subjected to different stimuli[74,75]. Although endocrine specification takes place before migration, maturation of endocrine cells occurs at islet level and coincides with islet vascularization[54,76]. There is a significant growth of islet cells that correlates with islet endothelial-cell proliferation in rats the first week after birth[76]. For instance, endocrine cells with higher proliferative capacity closer to blood vessels[76]. Furthermore, it is known that ECs are able to produce HGF which is a potent mitogen for beta cells and ECs[31,77]. Therefore, the endothelial signaling is essential for beta-cell maturation. For instance, immature beta cells are formed some days before birth and maturation occurs several days after birth[76]. During this period, immature beta cells express VEGF and start recruiting ECs to the vicinity of the islet to provide signals for further differentiation and maturation[32,78]. However, along with EC stimuli, another signals should also be considered. For example, hormones that can reach beta cells through the blood stream once the vascular network is established may have a role in cell maturation[79]. Apparently, endogenous insulin has a minor role for the glucose homeostasis before birth[80]. In this condition, insulin provided by the mother regulates glucose in the fetus[80]. This fact suggests that fetal beta cells are not mature enough at birth to maintain the glucose homeostasis and that further maturation can be promoted by ECs after birth.

ENDOTHELIUM ROLE IN PANCREATIC BETA-CELL FUNCTION

The role of ECs in β-cell function and pathology has been previously described[47,81-82]. It has been found that ECs from islets correspond to fenestrated endothelium[83,84]. Apparently the characteristics of islet endothelium differ from pancreatic exocrine endothelium and endothelium from other regions[47,83,85]. For instance, pancreatic endocrine capillaries have higher diameter than exocrine capillaries and endothelium from endocrine capillaries have 10 times more fenestrae that endothelium from exocrine capillaries[83]. These facts suggest that cell-cell interactions and signaling between endothelium and the surrounding cells are different even in the same organ. Pancreatic beta cells have polarity with an apical and basolateral membrane and insulin vesicles are more dense in the apical region close to ECs[47,86]. This aspect is very important when considering the ability of beta cells to release insulin into the blood stream. After islet transplantation, suitable EC-signals for polarization are crucial for appropriate insulin release into the capillaries. Islet ECs express common markers of ECs but one specific marker called the proteinase inhibitor and

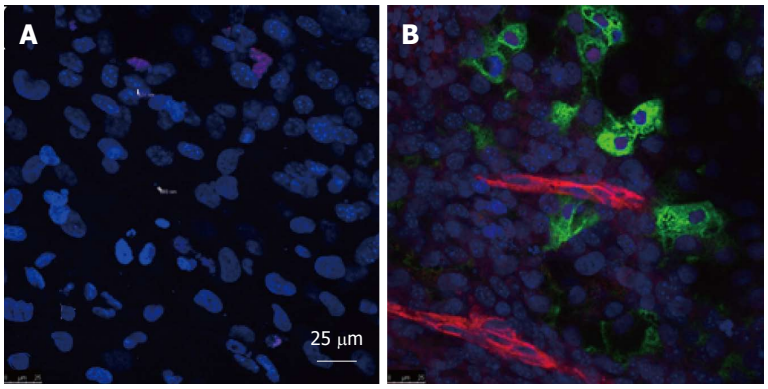

Figure 4 Analysis of blood vessels and insulin-producing cells in embryoid bodies obtained from human embryonic stem cell line H9. Human embryonic stem cells were cultured in suspension for 5 d to obtain embryoid bodies (EB). After attachment on coverslips for 24 h. Some EBs were cultured alone or together with human microvascular endothelial cells (HMECs). Then, after 20 d both groups of EBs were fixed and stained with with anti-proinsulin (green) (a marker for pancreatic beta cells), anti-CD31 (red) (a marker for endothelial cells), and DAPI (blue) (that stains the nuclei). A: EB cells cultured alone that do not show proinsulin or CD31 expression. In contrast with (B) EB cells co-cultured with HMECs at passage 14 in which we can find cells that express proinsulin in close proximity to cells that express CD31. HMECs did not stain positive for CD31 at the dilutions used indicating that the ECs are forming within EBs.

angiostatin factor α1-antitrypsin[70,84]. This marker of specific islet ECs can be absent in surrogate vasculature with deregulation of islet function. Apparently EPCs or islet ECs are important for islet revascularization after transplantation[87-89]. In this sense, any islet injury leads to islet restoration through recruitment of EPCs toward islet re-vascularization with specific islet ECs and beta cell function[87]. For instance, normoglycemia is improved in streptozotocin-treated animals after co-transplantation of EPCs and islets[87].

ENDOTHELIUM AND BETA-CELL REGENERATION *IN VIVO* AND *IN VITRO*

As a first approach to investigate the role of endothelium in β-cell differentiation, we studied the role of *in vivo* surrogate vasculature in mouse embryoid body (EB) differentiation using quail chorioallantoic membranes (CAM)[90]. We found that some cells expressed cardiotin, myosin heavy chain, collagen Ⅳ, CD34, CD31, and neurofilament. Although some epithelial cells appeared, no cells derived from endoderm were identified[90]. Then, studies using co-cultures between human microvascular ECs (HMECs) and mouse EBs were performed[52,53,91]. In these studies, ECs from human dermis were able to induce differentiation of mouse EBs to pancreatic progenitors and insulin-producing cells[52]. Furthermore, BMP-2/-4 were involved in this differentiation process as evaluated by the effects of agonists (recombinant BMPs), and specific antagonists of BMP bioactivities (Noggin, Chordin). BMPs are members of the TGF-β superfamily[92]. In addition to the effects of BMP antagonists, we explored the levels of phosphorylation of SMAD1, 5, 8 in cells that expressed proinsulin[52,53]. The role of BMPs in pancreas development has also been explored previously[72,93]. We demonstrated that HMECs or mouse dermis as well as mouse AECs expressed BMPs and that BMP-2 and BMP-4 increased the phosphorylation levels of SMAD1,5,8 in pancreatic progenitors and beta-like cells derived from mouse ESCs[52,53,94]. These findings together with previous works pointed out the important role of ECs in beta-cell differentiation *in vitro*. We recently have observed that when human ESCs (cell line H9) are co-cultured with HMECs, the formation of proinsulin positive cells takes place in about twenty days in close proximity to internal ECs without the use of additional growth factors (Figure 4).

In the model using mouse EBs, we observed that ECs promote up-regulation of BMPs within EBs[53]. However, the target cells that produce these BMPs are still unknown. One good candidate for these cells are mesenchymal cells since it has been demonstrated that ECs are essential to maintain dorsal pancreatic mesenchyme during pancreas morphogenesis that may promote pancreas differentiation within EBs[64] (Figure 5). However, further studies should be done to demonstrate that internal ECs are able to trigger beta cell differentiation through signaling to mesenchymal stem cells. At present, ECs can be generated *in vitro* from human induced pluripotential stem cells (hiPSCs) or human embryonic stem cells (hESCs) and this studies can be important to answer this question[95,96].

In this model, an excess of human microvascular ECs (HMECs) surround a human embryoid body which is composed of endoderm, ectoderm, and mesoderm cells. External ECs produce factors such as BMPs and other EC-derived factors that promote upregulation of endogenous BMPs in still unknown target cells (possibly mesenchymal or internal ECs). These BMPs together with other unknown factors may promote differentiation of multipotent cells (MC) toward beta-like cells (BC) and other cell lineages (OCL)[52,53].

ENDOTHELIUM AND BETA-CELL PATHOGENESIS

ECs play an important role for the pathogenesis of type 1 (T1DM) and type 2 diabetes mellitus (T2DM). ECs involvement in cellular diapedesis, inflammation,

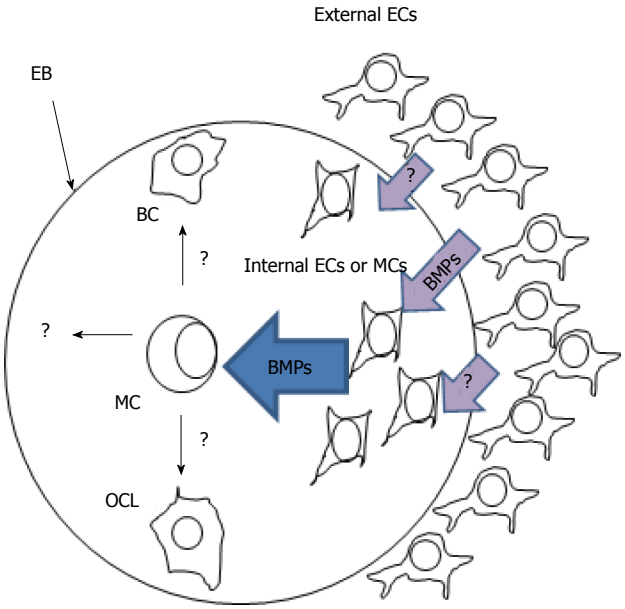

Figure 5 Diagram that explains the possible effects of external Endothelial cells toward *in vitro* beta-cell differentiation in human embryoid bodies. ?: Unknown factors, differentiation steps, or cell lineages; BMPs: Bone morphogenetic proteins; EC: Endothelial cells; EB: Embryoid bodies; BC: Beta cells; OCL: Other cells lineages; MC: Multipotent cell.

and Vessel fibrosis, which leads to islet dysfunction, has been demonstrated. In T1DM, Ecs allow infiltration of leucocytes[47]. Interestingly multiple diabetic complications relate to endothelial-cell function. The key to prevention or reversal of diabetes may rest in the recently emphasized role of endothelium in beta-cell differentiation and maturation *in vitro*[52,53].

In addition, it has been suggested that EC pathology can lead to islet dysfunction suggesting that ECs are essential to maintain islet function in adults[97].

CONCLUSION

In this review we focused in the essential role of endothelium for pancreatic endocrine differentiation, functional maturation, and islet dysfunction. ECs play a key role during the differentiation of the dorsal pancreas by maintaining the expression of transcription factors necessary for pancreas development including endocrine progenitors. Before birth, immature beta cells recruit ECs close to their microenvironment and these ECs provide signals for further maturation and function of pancreatic beta cells. In addition, ECs co-transplanted with islets have demonstrated to improve the engraftment of human islets. Finally, ECs are able to provide signals *in vitro* for derivation of functional beta-like cells from human pluripotent stem cells. Therefore, the study of interactions between EC and beta cells is relevant for future clinical applications in regenerative medicine.

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REVIEW

Are there new approaches for diagnosis, therapy guidance and outcome prediction of sepsis?

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Abstract

Beside many efforts to improve outcome, sepsis is still one of the most frequent causes of death in critically ill patients. It is the most common condition with high mortality in intensive care units. The complexity of the septic syndrome comprises immunological aspects - *i.e.*, sepsis induced immunosuppression - but is not restricted to this fact in modern concepts. So far, exact mechanisms and variables determining outcome and mortality stay unclear. Since there is no typical risk profile, early diagnosis and risk stratification remain difficult, which hinders rapid and effective treatment initiation. Due to the heterogeneous nature of sepsis, potential therapy options should be adapted to the individual. Biomarkers like C-reactive protein and procalcitonin are routinely used as complementary tools in clinical decision-making. Beyond the acute phase proteins, a wide bunch of promising substances and non-laboratory tools with potential diagnostic and prognostic value is under intensive investigation. So far, clinical decision just based on biomarker assessment is not yet feasible. However, biomarkers should be considered as a complementary approach.

Key words: Clinical decision-making; Biomarkers; Early prediction; Sepsis and mortality

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Core tip: Sepsis is a complex continuum of disturbed systems. Despite the presence of clinical consensus criteria, the early diagnosis especially in the perioperative setting is challenging. A magnitude of potential new biomarkers is tested for this purpose, but evidence is mounting that due to the complex nature of the syndrome, biomarkers are rather complementary tools for clinical decision making than "magic bullets". Moreover, biomarkers are also evaluated for therapy guidance, linking diagnostic results to an individual therapeutic regime. This review summarizes the developments in the biomarker field, aiming to provide an overview about current targets and their limitations.

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INTRODUCTION

The incidence of sepsis is still unreasonable high in critically ill patients and represents a major challenge in treatment. It is a common reason for admission to the intensive care unit (ICU). In European ICUs, sepsis and severe sepsis occur in 30% and 37% of the patients[1]. Gaieski and colleagues designate severe sepsis as the third most common cause of death in the United States after heart disease and malignant tumors[2]. A reason for the elevated incidence of sepsis in developed countries may be the high proportion of the elderly population[3].

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) with proven or probable infection of bacterial, fungal or viral origin[4]. Severe sepsis is characterized by additional existence of organ dysfunction, while septic shock is defined as sepsis together with the failure of the cardiovascular system to sustain adequate tissue perfusion[5] (Figure 1). Initially, the organism reacts with a proinflammatory immune response to the infectious stimulus. During the later course of disease, there is a co-existence between SIRS and compensatory mechanisms termed compensatory anti-inflammatory response syndrome (CARS, Figure 2). The resulting sepsis-induced immune suppression is characterized by a collapse of cellular immune response and an increased risk for opportunistic infections with high mortality[6]. Clinical signs of sepsis are unspecific and comprise general symptoms (*i.e.,* aberrances of body temperature, fluid balance, glucose metabolism or mental confusion), as well as laboratory indications of inflammation or signs of hemodynamic impairment and organ dysfunction[4]. Because of the high variability of symptoms and the pathophysiological complexity, clinical recognition and severity assessment remain difficult[7].

The therapy of septic patients represents a major challenge to physicians. To improve clinical management and outcome of critically ill patients, the Surviving Sepsis Campaign guidelines were published ten years ago and have been lastly revised in 2012[8]. However, despite modern resuscitating strategies and anti-infective therapy options morbidity and mortality remain notably high in septic disease.

The key to successful therapy remains the early detection of septic patients. Biomarkers may provide help for clinical decision-making and predicting sepsis-related outcome[9]. Therefore, beside commonly used substances like C-reactive protein (CRP) and procalcitonin (PCT), further biomarkers are needed. Additionally, in times with increasing prevalence of multidrug-resistant pathogens and a growing consumption of anti-infective drugs biomarker-guided strategies are of enormous importance[10]. The large involvement of organs and cell systems in the inflammatory response to an infection widens the number of putative biomarker candidates[11] (Figure 3).

BIOMARKERS OF INFLAMMATION

Cytokines and acute phase proteins

Triggered by inflammation, immune cells release a wide range of mediators, *i.e.,* interleukins or tumor necrosis factor (TNF-α), into circulation. These cytokines induce the production and secretion of acute phase proteins in the liver. Many of these substances have been investigated as potential sepsis-biomarkers. The following chapter reviews important markers of this group.

CRP and PCT

The most used biomarkers in clinical settings are the acute phase proteins CRP and PCT[4,10,12]. The synthesis of CRP in the liver is triggered by interleukin-6 (IL-6) in response to tissue damages, inflammatory and/or infectious stimuli[13]. There is a notable increase of CRP level 4-6 h after stimulation. These levels double every 8 h and peak 36 to 50 h after an infection/inflammation stimulus[14]. CRP measurement is cheap and rapidly available, but increases of CRP levels are unspecific, since they can be observed, *i.e.,* after surgery or trauma[15]. However, CRP is commonly used to screen early onset of sepsis in neonates[16]. High CRP levels correlate with disease severity and are discussed to represent the effectiveness of an antimicrobial therapy[17-19]. Although CRP assessment does not sufficiently allow to discriminate between infectious and non-infectious stimuli, a secondary rise in CRP level after 3 to 4 d after infection, surgery or trauma could be helpful for diagnosing septic complications.

PCT is a prohormone of calcitonin. The peptide precursor is released by parenchymal cells including liver, kidney and muscles cells as well as adipocytes in response to bacterial toxins. After exposition to those toxins, serum levels of PCT increase within 2 to 4 h[20] and a peak of PCT in serum can be detected after approximately 14 h[21,22]. PCT is seen as a specific biomarker for bacterial infection[23], although elevations of PCT in serum can also be observed under non-infectious conditions, such as trauma[24], major surgical procedures, pancreatitis and renal impairment[25-28]. Thus, the use of PCT as a diagnostic biomarker of sepsis is discussed in a couple of meta-analyses with conflicting results[29,30]. However, other trials investigated the potential use of PCT to guide antimicrobial therapies[31], *i.e.,* in patients with community-acquired pneumonia (CAP), acute exacerbation of chronic bronchitis and sepsis. A recent multicentre study demonstrated, that serum PCT levels are not an accurate indicator for ventilator-associated

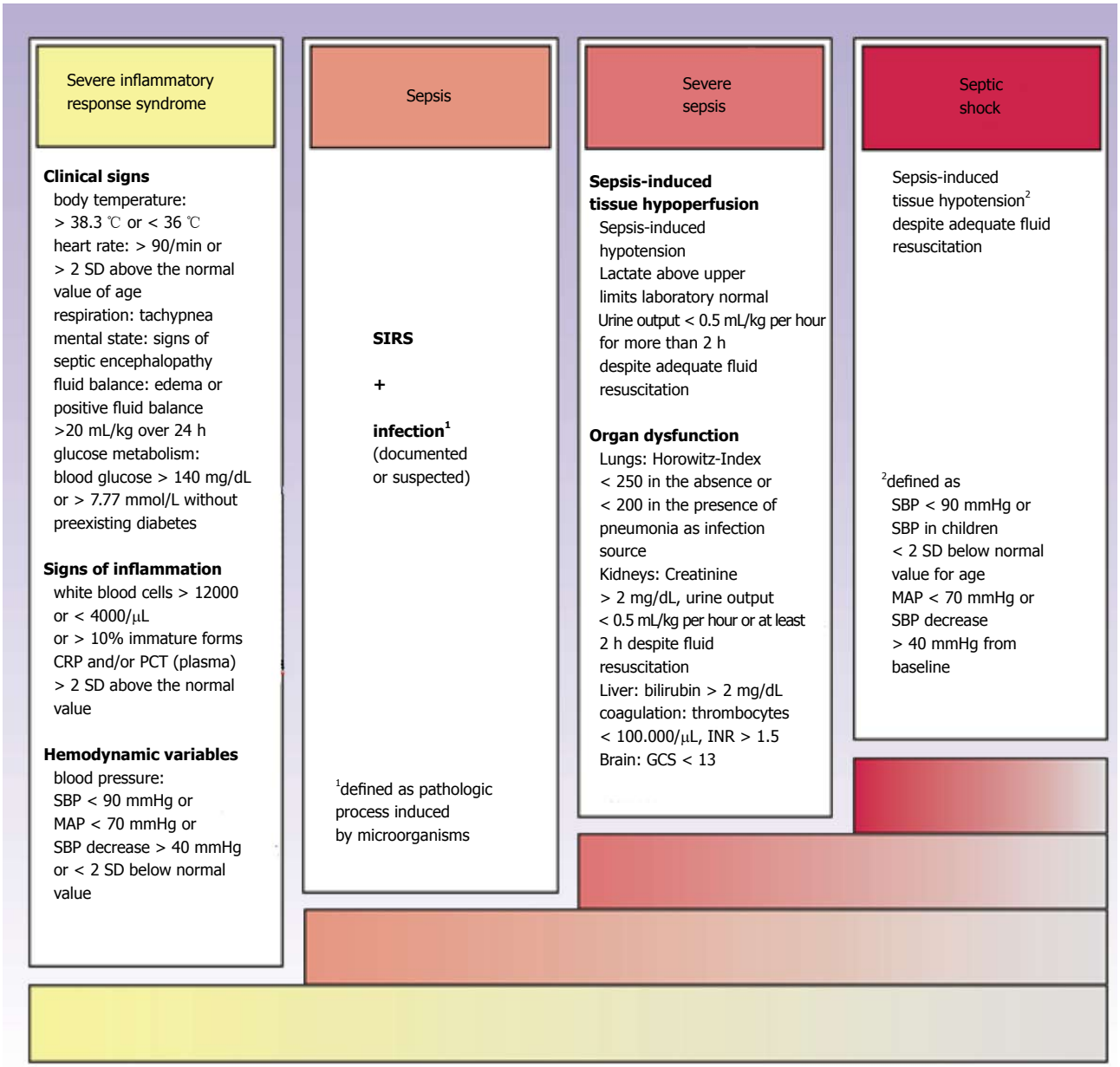

Figure 1 Diagnostic criteria of systemic inflammatory response syndrome, sepsis, severe sepsis and septic shock (modified from[5]). CRP: C-reactive protein; PCT: Procalcitonin; SIRS: Systemic inflammatory response syndrome.

pneumonia (VAP). Higher PCT levels were shown in CAP than in VAP[32]. Although PCT-guided antibiotic therapy is associated with a reduction in antibiotic usage and may reduce overall costs of care, an increase in hospital mortality of 7% under PCT guided conditions could not be ruled out by a meta analysis of Heyland *et al*[33]. Despite the association of high PCT and CRP levels in the onset of sepsis with poor outcome[34,35], the prognostic value of PCT is limited[10].

IL-6

IL-6 is secreted by macrophages and T cells to stimulate immune response which occurs during infection and after trauma, especially burns or other tissue damage leading to inflammation[36,37]. Compared to CRP or PCT, IL-6 levels peak 2 h after initiation of the inflammatory cascade. Based on this rapid increase, IL-6 was introduced as a biomarker of early sepsis in emergency units[38,39]. A multicenter study showed that IL-6 can predict survival on the 28th day after sepsis onset[40]. The value of IL-6 to distinguish between SIRS and sepsis is controversially discussed[41,42], since high levels can also be detected after trauma, surgery or in patients with autoimmune diseases[43-45]. Nevertheless, the IL-6 expression correlates with sepsis severity[10]. Further prospective and multicenter studies are required to elucidate the benefit of IL-6 and acute-phase proteins like CRP and PCT in diagnosis and risk-stratification of septic patients.

Pentraxin 3

The pentraxin superfamily comprises a wide range of

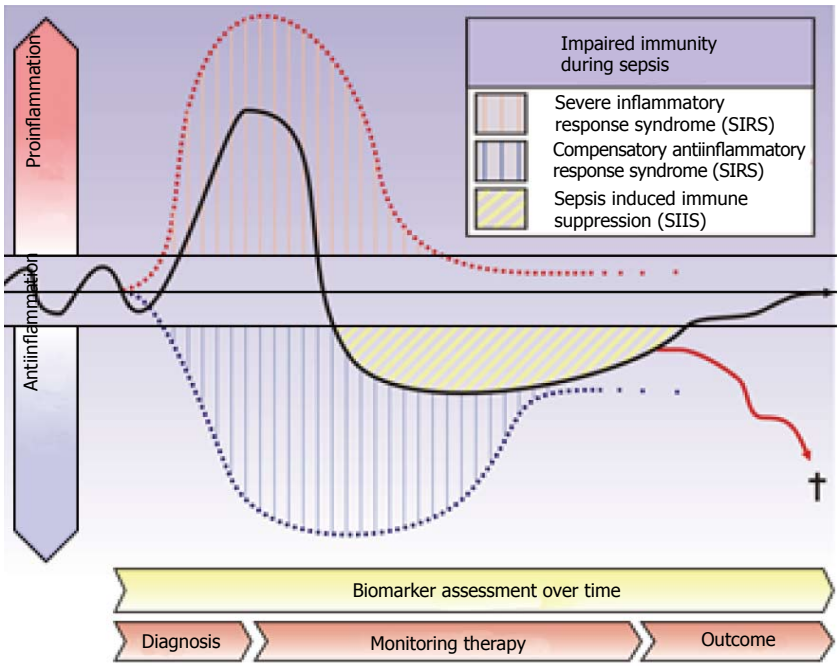

Figure 2 Simplified scheme of the impaired immunity during sepsis and the potential use of biomarkers: Initially, the body reacts to infectious stimuli with a proinflammatory immune response. Simultaneously, compensatory mechanisms are initiated to counteract the inflammatory process. The resulting net immune suppression is characterised by an increased risk of opportunistic infections. Beside C-reactive protein and procalcitonin, further biomarkers may be used in diagnosis, therapy-guidance and outcome prediction of sepsis.

proteins involved in the initial phase of the inflammatory response[46]. Prototypic long pentraxin 3 (PTX 3) is rapidly produced and released by various cells like mononuclear phagocytes, dendritic cells (DC), fibroblasts and endothelial cells[47-49]. It binds to specific patterns of fungi, bacteria and viruses[46,50,51] and induces the complement pathway[52]. A prospective study by Mauri and colleagues showed significantly elevated PTX3 levels in non-survivors compared to survivors over the first five days. In addition, PTX3 plasma levels were higher in the septic shock group than in patients with severe sepsis[53]. Concordantly, Bastrup-Birk and colleagues describe an association between disease severity and PTX3 levels. Furthermore, using ROC-analyses, moderate statistic values for PTX3 could be found to distinguish between patients with SIRS and healthy individuals[54]. Thus, PTX3 should be considered as a potential tool to monitor disease severity.

CELL-SURFACE RECEPTORS AND THEIR SOLUBLE FORMS

The expression of various proteins, *i.e.,* cell surface receptors, is up-regulated in activated immune cells. These receptors as well as their soluble forms occur as potential biomarkers in septic patients.

Soluble receptor for advanced glycation end products

The transmembrane receptor of advanced glycation end products (RAGE) belongs to the immunoglobulin superfamily. During inflammation, the receptor induces cell activation by initiating intracellular signalling cascades[10,55] resulting in rapid gene transcription[56]. RAGE is upregulated in various inflammatory diseases, such as rheumatoid arthritis[57,58], inflammatory kidney disease[59,60], arteriosclerosis[61] and inflammatory bowel disease[62]. The truncated form of the receptor, soluble RAGE (sRAGE), was demonstrated to be elevated in septic ICU patients at early onset of sepsis. There were also higher levels in nonsurvivors than survivors at day 28[63]. A multivariate analysis showed an association between sRAGE levels and mortality in acute respiratory distress syndrome (ARDS) patients, but not with severity of illness[64]. There is a relation between sRAGE and outcome of septic patients. To prove suitability of sRAGE as a prognostic marker, further clinical studies are needed.

Soluble triggering receptor expresses on myeloid cells-1

As a member of the immunoglobulin superfamily, the triggering receptor expressed on myeloid cells-1 (TREM-1) is strongly expressed on activated phagocytes[65,66]. A soluble form is released during bacterial or fungal infections and can be detected as biomarker in distinct body fluids[67]. After an induction period of less than 2 h, plasma levels of soluble triggering receptor expresses on myeloid cells-1 (sTREM-1) peak within 24 h[68]. However, a meta-analysis of 11 studies showed only moderate diagnostic performance in differentiating sepsis from SIRS[67]. Accordingly, Bopp and colleagues could not detect significantly different plasma concentrations between healthy controls and patients with SIRS, sepsis, severe sepsis or septic shock in the early phase of disease[63]. Since some trials report elevated sTREM-1 plasma levels during non-infectious states[69,70], the role of sTREM-1 as diagnostic biomarker remains uncertain.

sCD14-ST

Bacterial infections lead to complex formation of lipopolysaccharides (LPS), LPS binding protein (LPB) and the surface receptor cluster of differentiation 14 (CD14), which is located on the cell-surface membrane

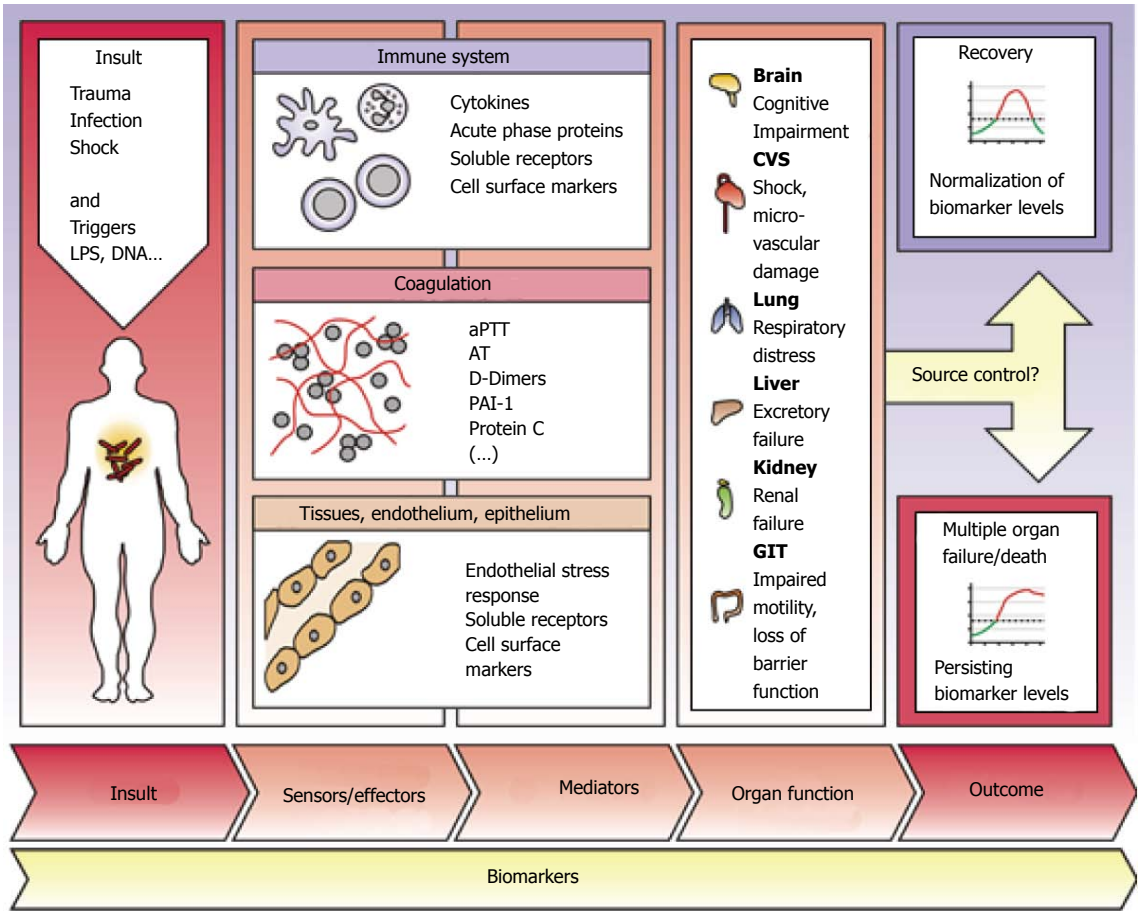

Figure 3 Use of biomarkers in sepsis: A wide range of biomarkers has been under intensive investigation to assist the clinician in diagnosis, outcome prediction and therapy guidance. LPS: Lipopolysaccharide; aPTT: Activated partial thromboplastin time; AT: Antithrombin; PAI-1: Plasminogen activator inhibitor-1; CVS: Cardio vascular system; GIT: Gastrointenstinal.

of phagocytes. During inflammation, a soluble form of this complex is further cleaved and termed soluble CD14 subtype (sCD14-ST = presepsin)[71,72]. Presepsin is a promising biomarker for diagnosing sepsis, severe sepsis and septic shock compared to other biomarkers such as PCT[73-76] and seems specific for infectious diseases[71]. High plasma levels in septic patients can be detected 6 h after early onset[72]. A recent study demonstrated that measurements of presepsin levels revealed diagnostic and prognostic value in patients with severe sepsis and septic shock within the first week of ICU treatment. Presepsin showed diagnostic value for sepsis, severe sepsis and septic shock at days 1, 3 and 8 after ICU admission in comparison to PCT, IL-6, CRP, white blood cells (WBC). It also revealed prognostic effort to prospect short- and long-term mortality[77]. These findings suggest that it may be used as a prognostic marker in early risk stratification.

Soluble urokinase-type plasminogen activator receptor

The urokinase-type plasminogen activator receptor (uPAR) is expressed on various cell types such as macrophages, endothelial and tumor cells. It participates in cellular immunity including migration, adhesion, angiogenesis, fibrinolysis and cell proliferation. After inflammatory stimulation, the receptor is cleaved from the cell surface by proteases and can be detected as soluble uPAR (suPAR) in urine, cerebrospinal fluid and blood[78].

Recent studies showed that high levels of suPAR are able to predict mortality in patients with diseases that are associated with inflammatory response. Additionally, the time course of suPAR seems to correlate with disease severity and the degree of organ dysfunction[79,80]. suPAR has gained growing interest because of its role as a predictor of disease severity in patients with bacteraemia[81]. In contrast to the prognostic value, the diagnostic performance of suPAR is limited[82].

Human leukocyte antigen-DR on circulating monocytes

Human leukocyte antigen-DR on circulating monocytes (mHLA-DR) is a major histocompatibility complex (MHC) class Ⅱ cell surface receptor and was originally defined as cell surface antigen. HLA-DR molecules are upregulated in response to inflammation[83]. Monocytes with low HLA-DR expression are unable to generate a proinflammatory response to microbial challenge or properly present antigens to T cells[84,85]. Decreased mHLA-DR levels could predict poor outcome and septic

complications after trauma, surgery, burn, pancreatitis and septic shock[86-89] but there is few knowledge about the underlying mechanisms[90].

A prospective randomised placebo-controlled trial used mHLA-DR to stratify the administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) in a small group of septic patients. After administration, there was an increase of monocyte release of tumor necrosis factor α (TNF-α). This biomarker-guided therapy appeared reliable and successful in reestablishing monocyte immunocompetence and shortening hospital and ICU stay[91,92]. The expression of mHLA-DR represents a valid indicator for monocyte function and should be tested in more clinical trials as it could be a reliable future marker for immunosuppression.

BIOMARKERS OF TISSUE DYSFUNCTION AND ORGAN FAILURE

The multiple organ failure (MOF) is the fatal end feature in the pathophysiology of sepsis. During severe sepsis or septic shock, development of organ dysfunction dramatically increases morbidity and mortality[93]. During the acute phase of sepsis or septic shock, circulatory failure is discussed as the predominant cause of death[94,95]. However, underlying mechanisms leading to organ failure are not fully understood yet. Since the breakdown of endothelial barrier functions is estimated to play an important role in this context[96], biomarkers of endothelial integrity as well as markers of cardiac injury are reviewed in the following.

Heart type fatty acid binding protein

Cellular as well as metabolic alterations during organ failure result in rapidly elevated blood levels of the heart type fatty acid binding protein (hFABP)[97]. The low molecular weight protein is predominantly detectable in myocardial cytoplasm and lung tissue. It participates in the uptake and transport of long chain fatty acids and is suggested as a promising biomarker of cardiac impairment[98-100]. Recent studies indicate, that hFABP can independently predict 28-d mortality and organ failure in patients with sepsis, severe sepsis or septic shock. Additionally, a correlation between plasma concentration and sepsis-induced myocardial dysfunction has been described and elevation of hFABP is associated with an increasing mortality rate[101-103]. hFABP may thus be a promising marker to identify high risk patients in the emergency department.

Troponin

Troponin T (TNT), a regulatory protein, is a highly specific marker for myocardial infarction or heart muscle cell death. Several trials accounted significantly increased TNT levels in patients with septic disease[104,105]. Contradictory, myocardial wall abnormalities, diagnosed by echocardiography, proved to be more specific in septic patients than increased troponin levels[10].

Natriuretic peptides

Many vasoactive hormones rise in sepsis[10]. Natriuretic peptides like atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) influence diuresis, natriuresis and vaso-relaxants[106]. ANP and BNP are predictors for cardiac dysfunction and are able to predict congestive heart failure[10]. Several trials demonstrated a correlation between high BNP levels and worse morbidity and mortality in septic patients combined with low ejection fraction[107,108]. High CNP levels have been detected in sepsis. Koch *et al*[80] showed significantly higher N-terminal pro-CNP levels in septic than in non-septic patients.

Cytokeratin 18

Apoptosis represents a major mechanism of cell death in patients with sepsis and multi-organ-failure[109]. Cytokeratins are proteins of keratin-containing intermediate filaments in epithelial tissue. It is cleaved by activated caspases (caspase-cleaved cytokeratin 18, cCK-18) and can be detected with the help of the monoclonal antibody M30[110,111]. The measurement of cCK-18 concentration conduce the dimension of apoptosis in critically ill patients[112-114]. There is a correlation between persisting high concentrations of cCK-18 in the early treatment of sepsis and 28 d mortality[115]. Several trials showed higher concentration of cCK-18 in septic patients than in trauma patients or healthy individuals[116,117]. Measurements of cCK-18 could be of valuable use in detecting risk of multi-organ-failure.

Angiopoetin-1 und -2

Microvascular disorders contribute to the development of MOF. The integrity and function of the endothelial barrier depends on the stability of distinct receptors and their ligands, *i.e.,* the Angiopoietin/Tie2 system. Regarding their antagonistic roles, especially the balance between Angiopoietin-1 and -2 (Ang-1 and -2) is of particular importance[118]. In a multicenter cohort study, low plasma levels of Ang-1 at admission as well as serially measured Ang-1 and -2 were associated with higher 28-d mortality in critically ill patients with severe sepsis[119]. Additionally, in patients with suspected infection, elevated Ang-2 plasma levels were detected during the first hour after admission and correlated with the development of severe sepsis and a higher mortality[120]. A number of trials investigate the value of an Ang-1/-2 ratio in predicting outcome of septic patients. This ratio was significantly elevated in children with severe sepsis and septic shock compared to control patients with or without SIRS or sepsis during the first 3 d on pediatric ICU[121]. Similarly, using Canonical Correlation Analysis, Wang *et al*[122] identified a strong correlation between the combination of Ang-1, Ang-2,

bicarbonate and disease severity or outcome of sepsis in children. Thus, the components of the Angiopoietin/Tie2-System are promising targets of further studies to improve outcome prediction in septic patients.

Midregional-pro-adrenomedullin

Posttranslational processing of the precursor peptide preproadrenomedullin results in the generation of the vasoactive, antimicrobial and anti-inflammatory peptide adrenomedullin (ADM)[123]. Systemic inflammation and sepsis leads to an increased release of ADM into circulation[124,125], but a short half-life and other difficulties in the measurement impede the clinical use[126]. However, another fragment termed midregional-pro-adrenomedullin (MR-proADM) seems to be a promising sepsis-biomarker. When blood samples are stored at 20 ℃, MR-proADM is stable up to 24 h. Thus, it seems more suitable for daily routine in the primary care setting[127]. The release of MR-proADM is increased during viral and bacterial infections. So far, the potential utility of MR-proADM to predict outcome of sepsis is controversially discussed in a number of trials. At admission to the ICU, single MR-proADM assessment may be useful to predict in-hospital mortality, although the statistic performance is not outstanding[128]. In patients with CAP, Suberviola *et al*[129] showed a correlation between severity of illness, outcome and the plasma levels of MR-proADM. However, this correlation could not be confirmed by another recent single-center study[130]. Further investigations are necessary to clarify this gap.

Endocan

Endothelial function, *i.e.,* the expression of surface proteins and the release of cyto- and chemokines, is crucial for migration of immune cells from circulation into tissues[131]. The proteoglycan Endocan, originally named endothelial cell-specific molecule 1, is expressed on the surface of pulmonal and renal endothelial cells. Proinflammatory cytokines like TNF-α and IL-β induce the expression and release into the bloodstream *in vitro*[132]. Various studies report altered levels of the proteoglycan in neoplastic diseases, since tumor-derived factors also regulate Endocan expression[133-136]. In septic patients however, elevated plasma levels are correlated with sepsis severity. Furthermore, significantly higher Endocan values can be detected in non-survivors at admission to ICU[137]. In addition, a recent study by Mihajlovic *et al*[138] determined a correlation between initially elevated Endocan levels and the development of sepsis induced organ failure over time[139]. Accordingly, in patients with ARDS, Endocan levels seem useful to predict mortality and multiple-organ dysfunction. Thus, Endocan may be a promising biomarker to predict disease severity and outcome of critically ill patients.

NON-LABORATORY BIOMARKERS

Besides numerous markers derived from plasma or other body fluids, a variety of non-laboratory tools like the assessment of body temperature, heart rate variability, blood coagulation or sidestream darkfield videomicroscopy can assist the clinician in diagnosis, outcome prediction and monitoring of septic patients. However, putative findings - although promising - are challenged by a variable user-dependency.

Body temperature patterns

A non-invasive technique to identify critically ill patients is recognition of body temperature patterns. Fever as a classic symptom of septic patients has minor sensitivity and specificity in relation to diagnostic expressiveness[140,141]. Drewry *et al*[142] showed that abnormal body temperature curves were predictive of the diagnosis of sepsis in afebrile critically ill patients. Analysis of temperature patterns may relieve the decision to antimicrobial therapy rather than absolute temperature values.

Heart rate variability

Besides its role in the regulation of various body functions, *i.e.,* heart rate, the autonomic nervous system participates in the complex host response to a systemic inflammation[143]. This link has been under intensive investigation. Measurement of the "heart rate variability" (HRV) is a promising technique to evaluate the autonomic cardiac regulation in patients with suspected sepsis. Although underlying mechanisms are still unclear, changes in HRV are associated with the appearance of systemic infections[144] and correlate with disease severity[145,146]. The measurement of HRV may be a promising tool to improve an early diagnosis of sepsis. In adult bone marrow transplant patients, alterations in HRV could be detected prior to the clinical diagnosis of sepsis[147]. Furthermore, in a prospective, observational study, initial detection of HRV changes in septic patients via electrocardiogram in the emergency department showed to be valuable in predicting in-hospital mortality[148]. However, since changes in cardiac function also depend on various cofactors, further research is needed to elucidate the role of HRV assessment in diagnosis and outcome prediction in patients with suspected sepsis.

Sidestream darkfield videomicroscopy

Impairment of the microcirculatory blood flow (MBF) is common in patients with sepsis and designated as an important step in the development of organ failure[149]. Sidestream darkfield videomicroscopy can be used to identify alterations of microvascular parameters, *i.e.,* the microvascular flow index, the perfused (small) vessel density or the proportion of perfused (small) vessels. Clinical studies using this technique in critically ill patients with cardiogenic or septic shock, but also in patients before and after cardiac bypass surgery, support the idea, that changes in MBF occur independent from macrocirculatory hemodynamic parameters[149-151]. In patients with sepsis, abnormalities in the sublingual MBF can be detected early in the septic

progress and correlate with disease severity[152,153]. Several trials investigated beneficial effects of various interventions to optimize sublingual MBF. However, these results should be interpreted carefully due to methodological limitations[154]. Further studies about the use of sidestream darkfield videomicroscopy in the clinical setting are necessary, but should consider potential observer bias, as reported by Sallisalmi *et al*[155].

Clot-Lysis-Index

Thrombelastometry is a proper tool for monitoring and therapy guidance of haemostatic dysfunction[156]. The Clot-Lysis-Index (CLI) measures the mechanical properties of forming a clot in whole-blood samples in a time-dependent modality. The clot firmness is measured at 30, 45 or 60 min. Recent trails have demonstrated significantly higher CLI in septic patients than non-septic patients and control groups. These changes have been detected in patients even before sepsis was diagnosed[157,158]. The CLI could be useful as a future tool for early diagnosis of critically ill patients.

CONCLUSION

In hospitalized patients, sepsis still belongs to the most frequent causes of death[1,94,159]. Unspecific predictive signs complicate an early diagnosis, and - when sepsis is successfully diagnosed - treatment strategies are rare. So far, therapeutic approaches are limited to fluid administration, antibiotics and the attempt to sustain or restore organ function[160]. Originally, sepsis was designated as a distinct inflammatory response to an infectious stimulus[161]. Modern concepts define sepsis as a syndrome and focus on the problem, that also non-infectious stimuli result in defence mechanisms and clinical signs, which in the end do not allow a discrimination between infectious or non-infectious origin[36].

Although sepsis is a major problem for the critically ill, it is not exclusively restricted to these individuals. The exact mechanisms and variables determining outcome and mortality are not yet fully understood. As a consequence, since there is no typical risk profile, it remains hard to define patients at risk, which hinders rapid and effective treatment initiation. Nevertheless, recent studies report a reduction of acute mortality of patients with sepsis, severe sepsis or septic shock[162]. However, survivors of the acute phase are confronted with a chronic dysfunction of organ systems with high mortality, recently termed as "persistent critical illness" (PCI)[96]. Thus, early diagnosis and a rapid treatment initiation are even more of crucial importance for the prognosis. Since the role of scoring systems in initial evaluation, monitoring and outcome prediction of septic patients is controversially discussed, biomarkers should be considered as complementary tools[163]. During the last decades, a variety of promising sepsis related biomarkers has been under intensive investigation. Some, *i.e.,* acute phase proteins, are already widely-used in clinical practice. So far, the Surviving Sepsis Campaign guidelines only recommend PCT evaluation over time to deescalate antibiotic therapies[5]. Although many efforts to find more specific biomarkers seem promising, evaluation and comparison of the results is limited by unstandardized development- and evaluation strategies.

The pathophysiology of sepsis is characterized by an impairment of various systems on cellular, tissue-specific or functional level. In contrast to the original idea of an explicit immunological dysfunction, sepsis is now seen as a more complex syndrome that is characterized by an "impaired homeostasis". This concept combines immunological aspects with a neuroendocrine dysregulation and barrier failures[96]. The complex nature of sepsis complicates the search for new treatment targets. Furthermore, physicians are confronted with various individual comorbidities and other influencing factors[164].

Lately, Brenner *et al*[165] showed the involvement of alternative mediators of cellular stress in the pathophysiology of sepsis. Methylglyoxal (MG), which is one of these highly reactive carbonyl species, is produced endogenously from the spontaneous degradation of triosephosphates (glyceraldehyde-3-phosphate and dihydroxyacetone phosphate) during glycolysis. The study identified MG as a better marker for the identification of patients with sepsis in comparison to routine diagnostic markers and furthermore, MG was shown to be an early predictor for survival in patients with septic shock. Hopefully, these findings will help to improve early recognition of sepsis.

Recent research approaches try to enlighten the (epi)genetic regulation of sepsis. Hopefully, new findings in this field may once help to improve early risk stratification. In this context, it seems obvious that no single biomarker can yet feature a high diagnostic value together with an outstanding sensitivity and specificity to predict outcome and to guide (antiinfective) treatment. Thus, the combination of markers can be beneficial. However, despite some interesting trials, a promising combination is still missing.

When discussing the use of sepsis-related biomarkers, the question remains which marker should be preferred in clinical use. Due to the complexity of sepsis, adequate interpretation of laboratory results is the basis for a reasonable biomarker assessment. Here, we reviewed the state of knowledge about some key biomarkers. Although many substances and methods seem promising, most of them are not yet established in the intensive care unit. In the daily routine, the assessment of biomarkers can be a complementary tool in clinical decision making. However, it should be restricted to accurately defined problems and pursue the objective to achieve direct benefits for the critically ill patient. Together with new findings in the epigenetic field, the concept of directly linking diagnostic

results to an individual therapeutic regime - also termed theragnostics[166] - may be the next step to improve the outcome of patients with sepsis. Thus, beside the need to find valuable diagnostic substances, biomarker-guided therapy approaches should gain further attention.

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REVIEW

Spotlights on immunological effects of reactive nitrogen species: When inflammation says nitric oxide

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Abstract

Over the last decades, nitric oxide (NO) has been definitively recognised as one of the key players involved in immunity and inflammation. NO generation was originally described in activated macrophages, which still represent the prototype of NO-producing cells. Notwithstanding, additional cell subsets belonging to both innate and adaptive immunity have been documented to sustain NO propagation by means of the enzymatic activity of different nitric oxide synthase isoforms. Furthermore, due to its chemical characteristics, NO could rapidly react with other free radicals to generate different reactive nitrogen species (RNS), which have been intriguingly associated with many pathological conditions. Nonetheless, the plethora of NO/RNS-mediated effects still remains extremely puzzling. The aim of this manuscript is to dig into the broad literature on the topic to provide intriguing insights on NO-mediated circuits within immune system. We analysed NO and RNS immunological clues arising from their biochemical properties, immunomodulatory activities and finally dealing with their impact on different pathological scenarios with far prompting intriguing perspectives for their pharmacological targeting.

Key words: Nitric oxide; Reactive nitrogen species; Post-translational modification; Immune cells; Immune diseases

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Core tip: Nitric oxide (NO) is a diffusible molecule, which is responsible for many physiological and pathological conditions. In this work we described some of its chemical characteristics and how it is generated. More, NO could rapidly react with other free radicals to generate different reactive nitrogen species (RNS). Indeed, we addressed the contribution of NO/RNS in different immune cells and how these reactive molecules are pivotal to control cellular responses focusing on inflammatory settings.

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INTRODUCTION

Nitric oxide (NO) is a diffusible molecule, which is involved in many different physiological and pathological conditions. It modulates blood flow[1,2], neural activity[3] and immune defence mechanism[4].

In biological systems, NO is mainly synthesised enzymatically starting from L-argine according to the following chemical reaction:

$$\text{Arginine} \xrightarrow[O_2 \rightarrow H_2O]{NADPH + H^+ \rightarrow NADP^+} \text{N-Hydroxyarginine}$$

$$\xrightarrow[O_2 \rightarrow H_2O + NO]{½\ NADPH + H^+ \rightarrow ½\ NADP^+} \text{Citrulline}$$

This reaction is catalysed by three different enzymes, identified in the '90s, encoded by different genes with different localization, regulation, catalytic properties and inhibitor sensitivity, called respectively neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3) nitric oxide synthase.

Genomic organization is similar among these isoforms suggesting a common ancestral progenitor and is composed by a bidomains structure (an oxigenase domain at N-term and a reductase one at C-term) with a intervening calmodulin (CaM) binding region between the two[5]. Calmodulin is necessary for the activity of all these enzymes, even though Ca^{2+}-dependence of NO synthesis distinguishes the NOS isoforms, with nNOS and eNOS having a much higher Ca^{2+} requirement than iNOS.

nNOS and eNOS are constitutively expressed among several cell types, including the endothelium, platelets, and neurons. Their function is mainly dependent on an intracellular calcium rise, even though other calcium independent mechanisms could impact on it, for example shear stress[6-10].

On the other hand iNOS is largely expressed only after induction by immunologic and inflammatory stimuli and its role in the direct protection against pathogens has been clearly demonstrated. For example, the requirement of iNOS for the eradication of *Mycobacterium tuberculosis* infection has been established[11] as well for other *Listeria monocytogenes*[12] and the protozoan parasite *Leishmania major*[13,14] in the '90s. Recent evidence has contributed to clarify mechanisms upon this immune response[15-17].

A fourth enzyme has been more recently characterised in rat liver and named mithocontrial NO synthase or mtNOS[18,19]. This latter enzyme has been shown to be constitutively active, calcium dependent and ascribable for mitochondria homeostasis and bioenergetics. Indeed, it has been shown mainly by the group of Ghafourifar that activation of mtNOS upon chemotherapeutic drug administration induces oxidative and nitrative stress, with consequent apoptosis of cells[20,21].

NO is not only the product of NOS enzymes but it is also generated in tissues by either direct disproportionation or reduction of nitrite to NO under the acidic and highly reduced conditions occurring in disease states, such as ischemia[22-24]. The biological significance of this alternative source of NO production consists in restoring physiological NO level when enzymatic production is uncoupled or dysregulated, as in atherosclerosis[25] or other inflammatory status[26].

NO AND RNS

Unlike reactive oxygen species (ROS), which are directed into the phagosome, NO is synthesised in the cytoplasm of the cell and diffuse rapidly across cell membrane[4]. Due to its chemical characteristics, NO could rapidly react with other free radicals such as $O_2^{-\bullet}$ to generate the highly reactive oxidant peroxinitrite ($ONOO^-$) and other reactive nitrogen species (RNS), which have been intriguingly associated with many pathological conditions such as chronic inflammation, atherosclerosis[27], diabetes[28], inflammatory bowel disease[29] and autoimmune diseases[30]. Peroxynitrite has multiple cytotoxic effects which are ascribable to aberrant generation of proteins, post-translational modification (PTMs) of the existing ones, DNA damage, activation of poly(ADP-ribose), mitochondrial dysfunction and cell death thus widely affecting transcriptional regulation, gene expression and cell signalling[31,32].

Among the several RNS-induced modifications, the prevalent reaction is the coupling of a NO moiety with sulfhydryl groups on proteins, yielding *S*-nitrosothiols. The most affected residue is tyrosine. Nowadays, the presence of nitro-tyrosine is commonly accepted as a hallmark of *in situ* inflammation and is associated with many different pathologies, spacing from atherosclerosis, genetic disorders to cancer[33-36]. However, NO alone is not capable of nitrating tyrosine thus the accumulation of 3-nitrotyrosine is the reaction product of the other RNS[37]. Moreover, under inflammatory conditions, tyrosine nitration may be dependent on the activity of myeloperoxidase, secreted by monocytes and polymorphonuclear neutrophils[38].

The biological significance of such modified residues lied in altered protein degradation[39], modification in protein properties[40,41], resulting signalling[42] and many other phenomena[43].

Tyrosine is not the only amino acid that is affected by the presence of RNS. Indeed, most of the amino acids containing aromatic rings could react with RNS and ROS. Among these, modifications of tryptophan were highlighted. Initially, nitration of tryptophan residues in proteins was assessed by means of proteomic assays, such as decreased in tryptophan-associated fluorescence and mass spectrometry, and was associated with decreased functionality of modified proteins[44]. However, the majority of the studies so far was unable to detect nitro-tryptophan derivatives *in vivo* due to the lack of good antibodies. This gap was recently filled by Ikeda

and collaborators, who developed an antibody specifically reactive with nitrated tryptophan residues[45]. This tool boosted the investigation of the presence of such modification *in vivo*. Ikeda and Yamakura in their studies identified 6-nitrotryptophan residues in extract of PC12 cells, suggesting that NO production drives physiological processes, such as differentiation and morphological changes. These claims were further supported by similar consequences observed upon tyrosine modifications[46-48].

NO, RNS AND INFLAMMATION

NO has been generally recognised as one of the key players involved in immunity and inflammation. In infectious conditions, NO displays antiviral and antimicrobial activities, both cytotoxic (tissue-damaging) and cytoprotective (tissue-preserving), acting as either an immunostimulatory (proinflammatory) or immunosuppressive (anti-inflammatory) agent. Most of NO-mediated immune effects have been demonstrated to be dependent on the activity of iNOS enzyme which, apart from releasing NO, provokes the depletion of local arginine (together with arginase) in macrophages or other host cells sustaining growth inhibition and death of the parasites[4]. Although it has been proposed that efficient pathogen killing require colocalization of iNOS with pathogen-containing compartments[49,50], the fact that NO can diffuse across cell membranes might allow for an antimicrobial activity at distance, even in those cases where NO acts in cells that do not express iNOS[51]. However, Olekhnovitch *et al*[52] have recently proposed a novel cooperative mechanism of collective NO production to sustain tissue-wide immunity during infections. Indeed authors provide evidence that the diffusion of NO from numerous phagocytes at the site of infection promotes equally effective parasite killing in NO-producing cells and bystander cells[52].

Further more, over the last decades, several reports attempted to definitively clarify NO appointment in the immune system[4]. Nevertheless, the plethora of heterogeneous NO-mediated responses precludes an univocal definition thus demanding additional investigations. Although NO activity mainly lies on its local spatio-temporal concentration, the phenotype and functional commitment of the immune cells, responsible for its generation, dramatically impact on its own activity. Additionally, given the intrinsic nature of this highly-diffusible uncharged gas, specific immune subsets become selective NO-targets even if not directly involved in its production.

This part of the review aims to provide intriguing spotlights on the role of NO in different immune cell subsets belonging to both innate and adaptive immunity.

Dogmatically, NO is produced by macrophages by means of iNOS activity which is transcriptionally primed by cytokines and microbial stimulation. The sustained generation of NO endows macrophages with cytostatic or cytotoxic activity against pathogens and tumour cells[53,54]. Although the production of NO by human macrophages remains controversial, growing evidence supports this notion providing data for the expression and activity of iNOS and eNOS isoforms in these cells[55].

Historically, macrophages were divided into two major categories (M1/M2) depending on their activation status and inflammatory attitude, even though this paradigm has been recently expanded to account for their enormous functional plasticity[56,57].

In 2000, Mills *et al*[58] ascertained a relevant metabolic discrepancy between M1/M2 subsets mainly due to a remarkable difference in arginine metabolism. Once differentiated, M1 or classically activated macrophages trigger Th1 immune response and secrete high amounts of NO to kill intracellular pathogens and to exert cytotoxicity towards tumour cells[59]. On the other side, M2 macrophages express high levels of arginase-I, which competes with iNOS for their common substrate L-arginine, thus preventing NO generation[60].

It is thus clear that NO represents a remarkable hallmark of macrophage activation states in pathological settings and that both macrophages and NO fulfil relevant and divergent roles in cancer biology. Mechanistically, it has been proposed that in the early stages of tumour progression, macrophages exploit high concentrations of NO and RNS to kill tumour cell clones. Later on, tumour-reprogrammed macrophages produce low levels of NO/RNS, which in turn promote cancer growth and spreading.

Weiss *et al*[61] postulated that NO, produced by intratumoural macrophages, represents the crucial determinant for the anti-metastatic potential of IL-2/α-CD40 immunotherapy. Conversely, iNOS expression and the coincident NO/RNS generation has been shown to contribute to the immunosuppressive attitude of myeloid-derived suppressor cells (MDSCs), a heterogeneous cell population associated with tumours[42,62,63].

The *dichotomous* activity of macrophage-derived NO definitely mirrors the aforementioned functional plasticity of these cells in response to environmental cues. The multifaceted role of NO in cancer will be deeply scrutinise in the proper paragraph of this manuscript named "NO, RNS and cancer".

NO represents a master regulator for the activity of other different immune subsets such as T lymphocytes, dendritic cells (DCs), natural killer cells (NKs) and mast cells. As for macrophages, NO potentially exhibits either positive or negative modulatory properties in all these subsets.

In cancer, it has been postulated that high concentration of NO impairs T cell functions by blocking the signalling cascade downstream of IL-2 binding the IL-2 receptors[64]. On the other side, lower concentrations of NO have been shown to promote Th1 differentiation by selectively up-regulating IL-12 receptor beta 2[65].

More recently, a cogent paper demonstrated that NO produced by iNOS in activated T cells impairs T_H17 cell differentiation trough the nitration of tyrosine residues in RORγt thus regulating IL-17 expression at the transcriptional level[66]. An interesting report designated NO as the driving force for the generation of a new subset of

regulatory cells (NO-Tregs) *via* the NO-p53-IL-2-OX40-survivin signalling pathway[67]. Nonetheless, by means of syngeneic mouse melanoma model, Jayaraman *et al*[68] postulated that iNOS, expressed by $CD4^+$ T cells, manifestly inhibits their commitment to Treg by blocking the release of TGF-β1.

Although compelling studies addressed the role of NO in T cell biology, NO contribution to the regulation of B cell activity remains unclear. Very recently, Giordano and colleagues shed light on the role of NO in regulating humoral immune responses. Indeed, authors suggested that NO generated by both inflammatory Mo-DCs and non-hematopoietic cells potentially regulate T cell-independent (TI)-2 antibody responses by inhibiting BAFF production[69].

DCs are the most powerful APCs of the immune system[70,71] representing the bridge between innate and adaptive immunity. In the canonical maturation pathway, microbial products trigger DCs activation, which leads to the production of large amounts of cytokines, especially IL-12 and IFN-α, driving the differentiation of naive T-cells into effector cells[72,73]. Moreover, DCs exposed to inflammatory cytokines rapidly activate other innate protective cells such as NK and NKT cells[74]. So far, the role of DCs as potential NO-producing cells has not been fully investigated and data concerning the impact of NO on DC maturation and functions are still debated. During the last years, several reports investigated this issue. Activated murine DCs do essentially express the iNOS isoform in response to cytokines or pathogen stimulation[75]. Conversely, the expression of iNOS and the production of NO during the commitment of human DCs are still debated. A recent report claimed that in the human immune system nNOS but not iNOS mediated NO synthesis is pivotal for the maturation and differentiation of these cells[76]. Nevertheless, the expression of iNOS as well as NO production clearly participate in the innate defence against intracellular pathogens[77].

Additionally, an interesting study by means of real-time metabolic flux analysis pointed out NO as the key metabolic regulator in inflammatory monocyte-derived DCs, expressing iNOS, in response to TLR stimulation[78]. In 2003, a groundbreaking publication firstly identified a new TNF/iNOS-producing (Tip)-DC subset in the spleens of *Listeria monocytogenes*-infected mice[79], whose role was recently clarified. Specifically, these cells act as sources of NO in a variety of infections clearly indicating that NO produced by DCs actively participates in both innate and adaptive immunity to pathogens[80,81].

NK cells are effectors of the innate immune system, instrumental for host defence toward infection from bacteria, virus and parasites[82]. Moreover, NKs actively participate in tumour surveillance and rejection of transplanted organs[83]. Nonetheless, the role of NO in NK cell activation is not completely understood. NOS isoform activity correlates with rodent NK cell-mediated cytotoxicity, as proved by both nitrite accumulation and pharmacological enzymatic interference[84]. In humans, the mechanism of NO production in NK cells needs to be clarified. It was proposed that the endogenous NO generation by active eNOS isoform prevents NK cells from activation-induced apoptosis, thereby maintaining cell fitness[85].

Mast cells (MCs) are widely distributed throughout the extravascular area in the body where they play versatile roles dealing with innate immunity, IgE-mediated allergy and inflammation. Indeed, they promote neutrophil phagocytosis, lymph node hyperplasia and can directly phagocytise and kill bacteria[86].

Data concerning NO/RNS generation by either rodent or human mast cells are still controversial. While investigating this topic in 2004, Swindle and colleagues concluded that activated rodent and human mast cells were unable to generate intracellular NO or to express iNOS[87]. According to this study, foregoing reports, indicating a potential NO-like activity in peritoneal mast cells, were misled by the presence of NO-producing macrophage in their cell preparation[88].

Conversely, a different group demonstrated the expression of NOS isoforms and production of NO by various MC populations including rat peritoneal MCs, human skin MCs (HSMC) and human mast cell lines (HMC-1 and LAD-2)[89,90].

Nevertheless, as reviewed for other immune subsets, it is well accepted that exogenous NO centrally impacts on mast cell functions. Exploiting a variety of either NO chemical donors or NOS inhibitors on both primary or mast cell lines, introductory reports in the field showed that NO blocks antigen-induced degranulation, mediator production and release[91]. Moreover, NO has been shown to promote CD8α up-regulation trough NO-cGMP pathway in rat peritoneal MCs. This remarkable event enlarges CD8 receptor sensitivity to alternative signals and coincidently boosts MC-mediated immune responses[92]. It is well known that during activation processes, MCs adhere to the extracellular matrix basically interacting with fibronectin, vitronectin, collagen type Ⅰ, collagen type Ⅳ, and laminin. NO impairs human MC adhesion to the matrix by an alternative mechanism that is mainly independent of the direct activation of sGC or RNS generation[93] (Table 1 and Figure 1).

NO, RNS AND AUTOIMMUNE DISEASE

In addition to promote an effective immune response in the control of infectious diseases, iNOS-mediated NO production may be involved in the dysregulation of immunity, playing a role in chronic inflammatory disorders. Autoimmune diseases could be considered as a chronic inflammatory status where the breakdown of immune tolerance, a complex process involving both genetic and environmental factors, is mainly caused by the post-translational modification of antigens. This occurrence results in the recognition of host proteins as "non-self" and indeed in the initiation of an adaptive immune response. Many inflammatory autoimmune diseases,

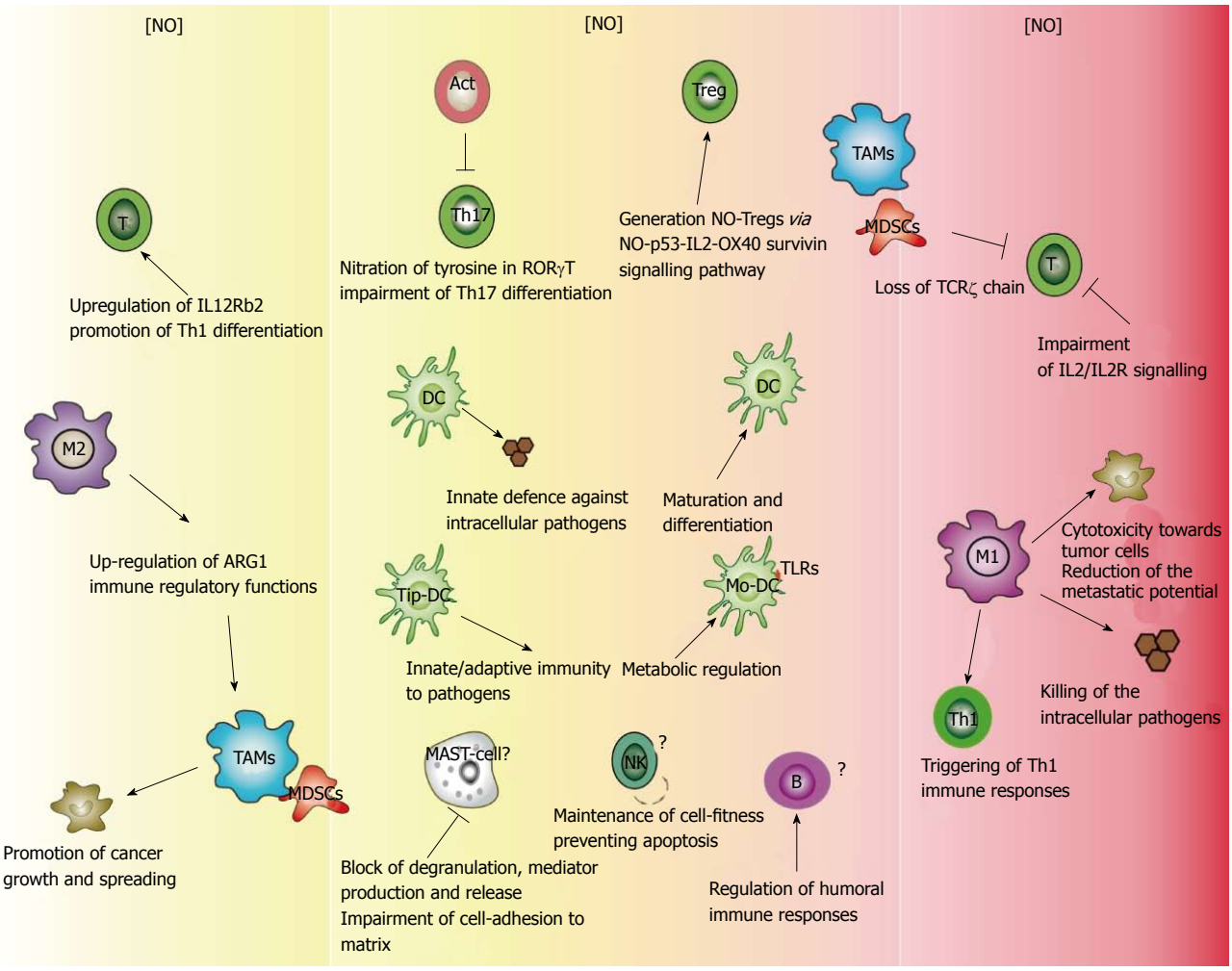

Figure 1 Distinctive effects of nitric oxide in immune cell subsets. Nitric oxide (NO) represents one of the key players involved in immunity and inflammation. The figure depicts the distinctive effects of NO within immune system, recapitulating its controversial behaviour. Low levels (left) could either favour Th1 response or immunoregulatory environment, while high levels (right) are either necessary for M1 macrophage effector function or could impair T cell activation. This opposite behaviour is more evident where NO concentration is poorly defined (middle), where NO sources and effects are more debated. Question marks tag open issues in the field. → or ┤ point out the effects of NO directly produced by the indicated cell on different targets. ← or ├ point out the effects of NO originated by different (not specifically indicated) sources on different targets. TAM: Tumour-associated macrophage; MDSCs: Myeloid-derived suppressor cells; IL: Interleukin; DC: Dendritic cell; NK: Natural killer cell.

which are accompanied by oxidative stress, exhibit an excess of reactive chemical species that are able to post-translationally modify proteins, potentially forming neo-epitopes[30,96-98]. These neo-epitopes may directly elicit an adaptive immune response or indirectly sustaining other mechanisms such as the molecular mimicry (a host antigen being "seen" as a "non-self" protein), the exposure of cryptic epitopes (exposure of amino acid sequences after changes in the three-dimensional structure of a protein), the epitope spreading (spreading of antigenicity from a given epitope to other parts of the same protein or other proteins) and the coupling of an autoantigen to an exogenous antigen[99,100]. Increased generation of neo-epitopes/PAMPs/DAMPs may therefore serve as a mechanism for increased uptake and presentation of autoantigens to the immune system, hence for example the accumulation of nitrotyrosine-containing proteins in tissues might induce an autoimmune response and sustain a chronic inflammatory reaction[96]. Indeed, murine models of systemic lupus erythematosus (SLE) showed abnormally high levels of RNS compared with normal mice and the systemic blockade of RNS production ameliorates the pathology[101]. Further and not surprisingly, elevated levels of anti-nitrotyrosine antibodies have also been measured in the synovial fluid of patients with rheumatoid arthritis and osteoarthritis[102] as well as in serum from patients with SLE[103-105]. This finding was also verified in patients with active lupus nephritis, who have higher levels of serum nitrotyrosine than those without renal disease, suggesting that overproduction of NO and its derived reactive species may have a pathological role in SLE and lupus nephritis[106,107].

NO, RNS IN DIABETES

Diabetes mellitus is a chronic disease characterised by elevated blood sugar levels resulting from either a lack of insulin production or resistance to insulin[108].

Table 1 Nitric oxide in immune cells

Cell compartment	Features	Ref.
Innate immunity		
Macrophage		
M1	High level of NO Expression of iNOS NO-mediated cytotoxicity	[55-59,61]
M2	Reduced level of NO Immune suppressive function	
Natural Killer cells		
	NO-mediated cytotoxicity NO-mediated cell fitness	[84,85]
Mast cells		
	NOS expression NO-mediated cell adhesion and function	[87,89-91,93]
Myeloid-derived suppressor cell		
	iNOS expression Immune modulating function	[62,64,94,95]
Dendritic cells		
	NOS expression Pathogen clearance	[72,73,75-77]
Adaptive immunity		
Lymphocyte		
T-cell	T-cell activation and function T-cell commitment	[64-68,72,73]
B-cell	Reduced level of NO T-independent antibody response	[69]

NO: Nitric oxide; NOS: Nitric oxide synthase; iNOS: Inducible NOS.

Hyperglycaemia, glucose autoxidation, accumulation of advanced glycosylation end products (AGEs), enhanced receptor for advanced glycation end product (RAGE) and angiotensin Ⅱ receptor type 1 (AT1R) signalling as well as elevated levels of free fatty acids and leptin, have been reported to contribute to elevated production of ROS and RNS in diabetic vessels and myocardium[109,110]. Several reports suggest a positive correlation between increased serum and/or vascular 3-nitrotyrosine levels, increased blood pressure and/or endothelial dysfunction in diabetic patients[111,112]. Additionally, high oxidative and nitrative stress in diabetes might induce oxidation and/or nitration of various insulin receptors in peripheral tissues, which may contribute to the development of insulin resistance[113]. Moreover, peroxynitrite injury has been implicated in the "metabolic memory" phenomenon, which refers to the therapeutic effects of intensive glycemic control achieved by early intervention in both experimental and clinical studies[28,114]. In diabetic hearts, the persistent myocardial oxidative and nitrative stress might also leads to dysfunction of important antioxidant defense mechanisms, such as the inactivation of superoxide dismutases and catalase and depletion of endogenous antioxidants, as metallothionein and glutathione[115,116] and dysregulation of important redox-dependent transcription factors [*e.g.,* NFE2L2 nuclear factor, erythroid 2-like 2 (Nrf2)][117,118]. However, peroxynitrite-induced protein nitration has been involved in the development of chronic diabetic peripheral neuropathy[119] and has been documented in peripheral nerve[120], vasa nervorum[121], spinal cord and dorsal root ganglion of streptozotocin-diabetic and obese mice[120,122], indicating that diabetes creates not just oxidative, but oxidative-nitrosative stress in the peripheral nervous system.

NO, RNS AND NEUROINFLAMMATION

As in other inflammatory disorders, NO plays a dual role in modulating neuroinflammation. On one hand, NO might induce apoptosis of auto-reactive T cells that enter the central nervous system (CNS)[123]; on the other hand, NO produced by iNOS within the CNS predominantly contributes to multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) pathogenesis[124]. Particularly, NO and peroxynitrite accumulation may affect the components of CNS causing lipid peroxidation and consequent damage of oligodendrocytes[125], disruption of blood-brain barrier integrity[126], activation of matrix metalloproteinases[127], with a block in axonal conduction[128] and finally promoting axonal degeneration[129]. Interestingly, recent findings on CNS of EAE rats demonstrated that iNOS-derived NO potently inhibits *CXCL12* gene expression in a p38-dependent manner *in vitro* and that inhibition *in vivo* of iNOS activity sustains *CXCL12* expression and protection of rats from EAE[130].

NO, RNS AND IBD

Early in the 1990s, various studies based on animal models as well as in humans, indicated that NO may be involved in gastrointestinal inflammation and that it may have a pathogenetic role in inflammatory bowel disease (IBD)[131]. Analysis of rectal biopsy specimens from patients with active ulcerative colitis showed higher concentrations of citrulline, the co-product of NO synthase, with respect to those from patients with quiescent disease or a normal histology, indicating that the increased biosynthesis of citrulline might be a consequence of NO synthase activity[132]. Additionally, NO produced following the up-regulation of iNOS in colonic epithelial cells has been closely associated with the initiation and maintenance of IBD[29]. Notwithstanding, the exact role of NO overproduction in intestinal inflammation remains obscure, since it has been reported that NO production plays a beneficial role in the acute nonspecific colitis settings. On the other hand however, in models of chronic colitis accompanied by a dysregulated immune response, where iNOS is persistently up-regulated, NO displays a detrimental activity on mucosal integrity[29]. High levels of NO from iNOS may in fact exacerbate the clinicopathological features of colitis by direct cytotoxicity, activation of neutrophils[133], vasodilatation and reduced smooth muscle tone[134].

NO, RNS AND CANCER

As already mentioned, NO and its derivative have been extensively associated with many different pathologies.

The connection between cancer and inflammation dates back to 1863, when Rudolf Virchow noted leucocytes in neoplastic tissues, suggesting that the "lymphoreticular infiltrate" reflected the origin of cancer at sites of chronic inflammation[135]. Moreover, smouldering inflammation increases the risk of developing many types of cancer, including bladder, cervical, gastric, intestinal, oesophageal, ovarian, prostate and thyroid cancer[136,137], thus representing one of the novel additional hallmarks of cancers[138]. As a result of chronic inflammation, tumour microenvironment harbours different corrupted resident or purposely recruited cells which exert conflicting functions establishing a peculiar cytokine milieu[139]. Among these, tumour-associated macrophage (TAM) and myeloid-derived suppressor cells (MDSCs) present in tumour overexpress iNOS and release oxidizing molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) which cause nitration and nitrosylation of components of the T cell receptor (TCR) signalling complex, and the loss of the TCR δ-chain, thereby inhibiting T cell activation through the TCR and contributing to tumour progression[95,140,141]. More recently, Molon *et al*[142], in 2011, have demonstrated that chemokine nitration prevents intratumoural infiltration of antigen-specific T cells, which remained confined at the edge of tumours being unable to reach the central core due to the nitration of CCL2, a master chemokine. Moreover, several investigators have reported the expression of iNOS by malignant cells or within the tumour microenvironment, both at mRNA and protein level. In breast carcinoma, an initial study suggested that iNOS activity was higher in less differentiated tumours and detected predominantly in TAMs[143]. Subsequently, other reports demonstrated that iNOS was also expressed by breast carcinoma cells and positively correlated with tumour stage[144] and microvessel density[145]. In addition to breast cancer, iNOS is markedly expressed in approximately 60% of human colon adenomas and in 20%-25% of colon carcinomas, while the expression is either low or absent in the surrounding normal tissues. Similar results were obtained for human ovarian cancer and melanoma. Other cancers that express iNOS are head and neck, oesophagus, lung, prostate, bladder and pancreatic carcinomas, brain tumours, Kaposi' s sarcoma, mesothelioma, and haematological malignancies[63]. Moreover, the eNOS has been found in both endothelial and tumour cells of breast carcinomas, and the nNOS has been detected in some oligodendroglioma and neuroblastoma cell lines. However, the role of NO in cancer biology has not been clearly elucidated yet, since various studies have shown that NO may either promote or inhibit tumour progression and metastasis. The net effect of NO in tumours seems to depend on the activity and localization of NOS isoforms, concentration and duration of NO exposure, cellular sensitivity and hypoxia/re-oxygenation process within tumour microenvironment[146]. In general, high concentrations of NO and RNS can arrest cell cycle (cytostatic effect) or induce cell death, whereas low concentrations may protect cells from apoptosis. In fact, generation of high levels of NO/RNS is a very effective tool to induce cell death, and macrophages use it as a major weapon in their arsenal against invading pathogens and tumour cells[147]. High levels of NO/RNS post-translationally modify death-related target proteins, as the death receptors of the TNF-α superfamily, and block respiration in target cells by affecting the mitochondrial respiratory chain and its outer membrane permeability and thus leading to the release of cytochrome c and apoptosis initiation[148,149]. Moreover, high NO concentrations, oxidizing and/or deaminating the DNA bases, result in DNA breaks, DNA base modifications or DNA cross-links, which cause mutations that may either activate oncogenes or deactivate tumour suppressor genes. In addition, NO/RNS-driven protein modifications such as S-nitrosylation or nitration may inhibit proteins belonging to the DNA repair systems, driving to genomic instability[150,151]. Importantly, DNA damages that cannot be repaired cause apoptosis induction, by the activation of DNA-damage-sensing proteins (*e.g.*, p53, PARP, DNA-PK, BRCA1, ATM)[152]. However, NO has been demonstrated to inhibit programmed cell death in endothelial cells and some liver cancer cell lines, mainly through S-nitrosylation of the active-site cysteine of caspases[153], thus perpetuating mutations and consequently sustaining transformation[154,155]. On the other hand, NO may induce apoptosis either via down-regulation of the anti apoptotic protein survivin, as observed in human lung carcinoma cells[156], or up-regulation of Fas expression, as shown in ovarian carcinoma cell lines, through the specific inactivation of the transcription repressor yin-yang-1, which binds to the silencer region of the Fas promoter[157]. Besides enhancing cytotoxicity, NO plays a role in angiogenesis and metastasis since several reports showed a positive correlation between NOS expression/activity in tumour tissues and lymphatic metastasis in head and neck, thyroid, breast, stomach, gallbladder cancers[146,158] and melanoma[159]. As under physiologic conditions, NO maintains blood flow by dilatation of arterial vessels, promotes perivascular cell recruitment and vessel remodelling and maturation within tumours[146]. NO exposure of A-431 squamous carcinoma cells and MDA-MB-231 breast cancer cells was, in fact, able to induce VEGF-C expression, which mediates lymphangiogenesis and metastasis[160]. However, NO may also inhibit the aggregation of platelets through a cGMP-dependent mechanism, preventing aggregates formation with tumour cells, which may facilitate their adhesion to vascular endothelial cells and haematogeneous dissemination[161]. Finally, as previously mentioned, NO/RNS can suppress tumour-specific adaptive immunity through several mechanisms. One mechanism involves the inhibition of phosphorylation, and thereby the activation of important signalling proteins in the IL-2-receptor pathway [including Janus activated kinase 1 (JAK1), JAK3, STAT5, extracellular-signal-regulated kinase (ERK) and AKT] in T cells[162]. Additionally, NO promotes tumour immunosuppression, by affecting the stability of IL-2 encoding mRNA and the release of IL-2 by activated human T cells[163]. Finally, NO/RNS may dampen anti-

Table 2 Nitric oxide and its effect in pathologies

Disease	Pathogenesis	Ref.
Autoimmune diseases		
	Appearance of neo-epitopes Disruption of physical barriers Amelioration of pathological status	[29,30,96-98,101,123, 126,130]
Metabolic disease		
	Deregulated accumulation of proteins or their abnormal modification	[109,110,115,119,120]
Cancer		
	Establishment of an immune suppressive environment Alteration in DNA repair mechanisms	[140-142,147,151,152, 155,157,160]

tumour immunity through post-translational modifications of key proteins for T cell activation, such as CD8 and TCR complex α/β chains molecules[42] and T lymphocyte recruitment to the tumour site, such as the chemokine CCL2[142]. Thus, interfering with NO/RNS production within tumour microenvironment may represent a promising successful strategy to implement the efficacy of anti-tumour therapy alone or, even better, in combination with conventional chemotherapy, radiotherapy, photodynamic therapy and immunotherapy approaches[164-167] (Table 2).

CONCLUDING REMARKS

Over the last decades, the role of NO in the immune system has been extensively reviewed. While investigating NO-mediated responses, a number of reports argued for either a NO-stimulatory or -inhibitory activity in distinctive immune events. Nonetheless, the generation of NO in several immune cell subsets remains still controversial thus demanding additional studies.

So far, a general consensus in the field has been achieved highlighting the indisputably role of this diffusible mediator in shaping immune activities.

This manuscript aims to provide spotlights on NO-moulded biology specifically focussing on its pivotal participation in distinctive inflammatory programs. Indeed throughout this review, we scrutinised the role of NO in selective scenarios starting with the description of its biochemical properties, immunomodulatory activities and finally dealing with its remarkable impact on different pathological settings. Collectively all the aforementioned investigations pointed out the relevance of NO-mediated effects in the regulation of either innate or adaptive immunity.

Additionally, the combination of NO with other reactive species originates RNS, which are actively involved in several pathological conditions such as chronic inflammation, autoimmune diseases and cancer. Basically, RNS generate and amplify distinctive inflammatory circuits by affecting protein structure and functions, gene expression, cell signalling and cell death.

Hence, this manuscript also emphasises the duplicity of NO-mediated responses in distinctive immune cell subsets. This dichotomous attitude apparently hinders the identification of NO as a foolproof target thus preventing the identification of feasible therapeutic strategies that could be rapidly delivered to the clinic. Nonetheless, we believe that the plasticity of NO signals could be potentially exploited for the development of new focussed pharmacological approaches.

Of note, RNS-mediated PTMs potentially represent a novel marker for monitoring the efficacy of therapy during disease treatment or in the follow-up care.

This pursuit requires a thorough understanding of NO/RNS biology in the context of the immune system thus opening the way for intriguing investigations.

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REVIEW

Obstructive sleep apnea syndrome and cardiovascular disease: The influence of C-reactive protein

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Abstract

Obstructive sleep apnea syndrome (OSAS) is a common medical condition, associated with atherosclerosis and cardiovascular disease (CVD). The underlying pathophysiologic mechanisms of this association have not been completely understood and may be multifactorial in origin. A number of studies suggest that inflammatory processes have emerged critical in the pathogenesis of CVD in OSAS. A range of circulating inflammatory molecules has been identified and measured, with a view to assess inflammation and predict vascular damage risk, such as plasma cytokines, adhesion molecules, and C-reactive protein (CRP). CRP is a relevant marker worthy of further study, because not only is elevated in patients with OSAS, but also is rapidly becoming a risk factor for cardiac disease. Furthermore, in selected OSAS patients, aggressive treatment of the disorder may lead to retarding or even improvement of CVD progression. However, still there is a debate on the true correlation between CRP and OSAS, as well as the clinical effect of any reduction after OSAS treatment. Further research is required to define those OSAS patients who will have a considerable reduction with treatment, as well as to understand the significance of the interaction between cardiovascular risk factor and CRP reduction in patients with OSAS.

Key words: Sleep apnea; Cardiovascular; C-reactive protein

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Core tip: Obstructive sleep apnea syndrome (OSAS) is a common medical condition, associated with atherosclerosis and cardiovascular disease (CVD). A number of studies suggest that inflammatory processes have emerged critical in the pathogenesis of CVD in OSAS. C-reactive protein (CRP) has been the most studied inflammatory protein to date and a frequently used marker to predict the occurrence of CVDs. Unfortunately, the question still remains if CRP is truly related to OSAS, as well as the clinical effect of any reduction after treatment of OSAS.

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INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is a common medical condition, characterized by repetitive episodes of upper airway obstruction that occur during sleep[1]. These intermittent episodes are leading to disruption of normal ventilation and sleep architecture and ultimately through a range of pathophysiological mechanisms in cardiovascular disease (CVD). Indeed OSAS, when not correctly treated, has been associated with higher fatal and nonfatal cardiovascular events[2]. It is worth noting that the cardiovascular consequences of OSAS may develop even in the absence of the traditional cardiovascular risk factors[3]. Therefore, clinicians should be aware that OSAS has emerged as an independent risk factor for CVD[4].

Several mechanisms are involved in the association between OSAS and CVD, such as enhanced sympathetic activity, oxidative stress, systemic inflammation, and endothelial dysfunction which promote atherogenesis. Atherosclerosis could be one of the mechanisms connecting OSAS to CVD. There is growing evidence that the underlying inflammatory process plays a crucial role in all stages of the atherosclerotic disease process, with established CVD seen as the end of a long process of inflammation-mediated atherosclerosis[5,6]. Since inflammation has a key role in the development of CVD it is a common sense to assume that OSAS may contribute to CVD through an inflammatory mechanism. Therefore, evaluation of circulating biomarkers of inflammation could be a useful risk assessment tool for identifying patients with cardiovascular events.

A range of circulating inflammatory molecules has been identified and measured, with a view to assess inflammation and predict vascular damage risk, such as plasma cytokines, adhesion molecules, and C-reactive protein (CRP). Of these markers, CRP, an acute phase reactant synthesized in the liver, is one of the most widely investigated biomarkers of low-grade inflammation in CVD. A number of studies have demonstrated that CRP is a significant risk factor for atherosclerosis and higher CRP levels, even in the high normal range (0.2 to 1.5 mg/dL) are associated with high cardiovascular morbidity and mortality in individuals with and without known CVD[7-10]. In patients with OSAS elevated levels of inflammatory markers, such as CRP, have been found; however there is a debate on the true relationship between CRP and OSAS.

This short review will try to highlight the most clinically relevant updates on the relationship between OSAS and CRP and how this relationship could contribute to CVD. It cannot judge all newly-available information, but information from the literature considered to be of sufficient primary care interest has been summarized.

OSAS AND INFLAMMATORY ACTIVATION

OSAS is characterized by cyclical episodes of hypoxia and reoxygenation that can provoke oxidative stress due to reactive oxygen species production and inflammatory mediators activation[11]. In addition, these alterations can turn on nuclear transcriptional factors, including nuclear factor-B, which induce production of inflammatory mediators, intracellular and vascular cell adhesion molecules[12]. All the above could facilitate vascular endothelial damage and atherogenesis[13-15]. In this way, intermittent hypoxia may lead to atherosclerosis and, ultimately, the cardiovascular consequences of OSAS.

A range of inflammatory molecules has been localized to the atherosclerotic plaque[16-18]. Among them, CRP and interleukin-6 (IL-6) are the most widely studied in CVD.

OSAS AND CRP

Several authors have studied the relationship between apnea-hypopnea index (AHI) and CRP levels in OSAS patients, but the results are contradictory (Table 1). Factors such as sample size, statistical methodology and study design could be responsible for this disparity. Most of the studies to date[19-22] presented elevated CRP levels in OSAS patients; however the role of obesity and sleep duration in this CRP elevation has been questioned by other studies[20,23]. Therefore, as the role of CRP as a risk factor in OSAS is open to discussion, one could speculate that CRP's value as a special marker of OSAS-related cardiovascular risk is attenuated. However, a recent meta-analysis has shown that patients with OSAS had a statistically significant higher level of CRP and this effect was positively influenced by OSAS severity[24].

IS CRP PART OF THE LINK BETWEEN CVD AND OSAS?

Cardiovascular complications represent a considerable part of OSAS complications. OSAS is an independent risk factor for CVDs, such as hypertension, arrhythmias, pulmonary hypertension, coronary artery disease, congestive cardiac failure and cerebrovascular events[25,26].

Although a matter of debate, the role of OSAS in the pathogenesis of hypertension is strongly suggested[11,27], involving markers or pathways indicative of systemic inflammation, such as CRP. Furthermore, in animal studies intermittent hypoxia has been shown to produce hypertension[28,29].

Cardiac arrhythmias appear to be a common in OSAS patients; still their true prevalence remains

Table 1 Previous studies on the relationship between obstructive sleep apnea syndrome, C-reactive protein and other inflammatory markers

Ref.	Study population	Inflammatory markers	Findings
Chami *et al*[78]	355 OSAS patients 545 controls	CRP, fibrinogen, IL-6, TNF-α, TNF-R2, CD40-ligand, P-selectin, ICAM1 MCP-1 after PSG	AHI was associated with CRP, IL-6, fibrinogen, ICAM, and P-selectin but not with TNF-α, TNF-R2, CD40-ligand, or MCP-1 levels The associations were substantially attenuated, and remained statistically significant only for IL-6, after adjustment for BMI
Kurt *et al*[79]	78 OSAS patients 20 controls	CRP, hemoglobin, RDW, MPV, PDW, and white blood cell	The severity of OSAS was not correlated with CRP, MPV, and RDW
Svensson *et al*[80]	136 OSAS patients 262 controls	CRP, TNF-α, IL-6, myeloperoxidase and lysozyme	The levels of CRP, IL-6 and lysozyme were significantly higher in subjects with AHI > 15 compared with subjects with lower AHI In multiple linear regressions adjusting for age, waist circumference and smoking, independent correlations between ODI and inflammation were found for IL-6 and TNF-α
Lin *et al*[38]	109 OSAS patients 35 Controls	Serum glucose, and lipid profile comprising total cholesterol, high density lipoprotein-cholesterol, low density lipoprotein-cholesterol and triglycerides hs-CRP	CRP and metabolic score significantly increased with the aggravation of OSA severity OSA was independently associated with sum of metabolic components and CRP
Guven *et al*[48]	47 OSAS patients 29 controls	CRP	The serum CRP levels were significantly higher in the OSA group than in the control group In multiple regression analysis, elevated hs-CRP levels were associated with AHI which was independent of obesity
Kokturk *et al*[20]	94 OSAS patients 57 controls	CRP and conventional cardiovascular risk factors such as total cholesterol, low-density lipoprotein, high-density lipoprotein, very low density lipoprotein, and triglycerides	CRP levels were significantly elevated in the OSAS group compared to the non-OSAS group CRP levels were independently associated with AHI
Shamsuzzaman *et al*[19]	22 OSAS patients 20 controls	CRP	CRP levels were independently associated with OSA severity
Punjabi *et al*[21]	69 OSAS patients	CRP	A strong association was found between degree of SDB and serum levels of CRP, with or without adjustment for age and several measures of adiposity. Moreover, an independent association between serum CRP levels and nocturnal hypoxia was also observed, whereas no association was noted with parameters of sleep architecture
Yokoe *et al*[22]	30 OSAS patients 14 controls	CRP and IL-6	CRP and IL-6 and production of IL-6 were significantly higher in patients with OSAS than in obese control subjects. The severity of OSAS and BMI were independently related to levels of CRP, whereas BMI and apnea-related nocturnal hypoxia were independently related to levels of IL-6 in patients with OSAS
Hayashi *et al*[23]	60 OSAS patients 30 controls	Hsp-70, TF, MCP-1, hs-CRP, hepatocyte growth factor and plasma vascular endothelial growth factor	Serum CRP, TF, MCP-1 and Hsp-70 levels were significantly higher in OSAS compared with control subjects However, the best correlation with serum CRP levels was BMI and it was the most significant determinant for CRP
Schiza *et al*[65]	528 OSAS patients	CRP	CRP levels were independently associated with OSAS severity

AHI: Apnea hypopnea index; BMI: Body mass index; ODI: Oxygen desaturation index; OSAS: Obstructive sleep apnea syndrome; CRP: C-reactive protein; hs-CRP: Highly sensitive C-reactive protein; IL-6: Interleukin-6; Hsp-70: Heat shock protein-70; TF: Tissue factor; MCP-1: Monocyte chemotactic protein-1.

unknown. The severity of OSAS, as shown in most studies, is independently associated with elevated markers of systemic inflammation, including CRP. CRP is directly associated with an increased atrial fibrillation burden[30] and strongly and independently associated with occurrence of heart failure[31,32]. Furthermore, serum levels of CRP were significantly increased in patients with OSAS and an acute cardiovascular event[33]. In patients with coronary artery disease on current optimal medications for secondary cardiovascular risk reduction, highly sensitive CRP was significantly correlated with the severity of OSAS, suggesting that OSAS could activate vascular inflammation in these patients despite current best practice medications[34]. Moreover, OSAS is independently associated with increased levels of CRP in patients with acute ischemic stroke[35]. Therefore, CRP could be a part of the pathophysiological pathway linking OSAS to stroke.

CONFOUNDING FACTORS

Role of obesity

In patients with OSAS, the increased CRP levels are still under critisism, because of the impact of confounding

factors such as obesity, other CVDs and medications. Although, several studies have indicated independent associations between CRP levels and OSAS[19-23,36-38], others do not demonstrate significant correlations after adjustment for associated confounding variables[39-42]. It is possible that OSAS alone may not contribute substantially to CVD since multiple factors have been implicated in the occurrence of CVDs in high-risk patients. Among them, metabolic parameters are important factors, since metabolic syndrome is closely related to cardiovascular morbidity and mortality[43,44].

One should keep in mind that obesity is prevalent in patients with OSAS, and it has been shown that elevated CRP levels are significantly and independently correlated to high BMI[45]. Similarly, other investigators found that CRP levels in OSAS strongly correlate to obesity and not to OSAS severity[42,46,47]. Therefore, based on these studies as nonobese OSAS patients don't demonstrate statistically increased levels of CRP, any CRP elevation noted in obese OSAS patients may reflect chronic inflammation attributable to obesity and not to chronic hypoxia due to OSAS. Furthermore, it is likely that by interacting with obesity, OSAS further increases systemic inflammation and therefore increases the CRP levels in obese OSAS patients compared to obese without OSAS.

Recent studies, however, have shown a close correlation of CRP level with OSAS severity meaning that the higher the AHI, the higher the levels and CRP elevation seems to be independent from visceral obesity in patients with OSAS[4,19,21,24,34,38,48-54]. The above studies imply that factors other than adiposity may have a central role for the proinflammatory status observed in OSAS.

ROLE OF GENDER

It is worth noting that gender has been considered as a variable in CRP evaluation[55]. A number of studies have also presented higher CRP levels in women compared to men[19-23,36,37,39,51,56-59], probably due to a greater degree of adiposity in women[56,59]. Previously we have shown that moderate to severe OSAS females patients had higher although not statistically significant CRP values, compared to matched males[60], and more recently Yardim-Akaydin *et al*[61] showed statistically significant increased values of CRP, in female OSAS patients.

EFFECT OF EXERCISE

There is no agreement in the scientific literature regarding the correlation between exercise and CRP levels. Cavagnolli *et al*[62] evaluated the effects of aerobic exercise on CRP in non-obese patients with OSAS and found that CRP levels were not elevated and did not change after 2 mo of physical exercise.

Clinical implications

As CVDs are detrimental to human health, medical practitioners seek to predict the development of cardiovascular events before they occur. For this reason, the evaluation of CRP as a circulating biomarkers of inflammation has become a useful tool for stratifying patients at high risk for future cardiovascular events. CRP is a relevant marker worthy of further study, because not only is elevated in patients with OSAS, but also is rapidly becoming a risk factor for cardiac disease. Furthermore, in selected OSAS patients, aggressive treatment of the disorder may lead to retarding or even improvement of CVD progression.

CRP has also captured researchers attention, because tests measuring their levels are widely available even in general hospitals. High-sensitivity assays to detect low concentrations of the protein have become available, permitting CRP levels measurement as low as 0.007 mg/L[63]. Contrary to other cytokines, CRP levels are quite stable in the same individual over 24 h and may reflect the level of inflammatory response, without concern for time of day[6]. Furthermore, the American Heart Association approves the use of CRP in risk factor estimation in adults without CVD.

EFFECT OF POSITIVE AIRWAY PRESSURE THERAPY ON LEVELS OF CRP

Positive airway pressure (PAP), mechanically splints the upper airway, thus preventing soft tissue of upper airway from narrowing and collapsing during sleep, has been considered as the most effective treatment for OSAS[64]. Nevertheless, studies concerning the effects of PAP therapy on CRP levels are also contradictory. Among those, most studies had small sample size, which is prone to a false or spurious conclusion.

Our group and others have shown a gradual decrease of CRP levels with effective PAP therapy, which could subsequently improve cardiovascular morbidity associated with OSAS[19,65-69], whereas others found that PAP therapy did not significant change CRP levels, regardless of PAP therapy duration[39,42,65,70-74]. Furthermore our study[60] showed that after PAP therapy CRP was decreased more slowly in females compared to matched for OSAS severity males. CRP levels in females remained unaltered for the first 3 mo, irrespective of effective PAP treatment, while on the contrary males presented a significant fall in CRP over that period. At 6-mo, a significant decrease was observed in all patients who used PAP, while CRP values approached those of subjects without OSAS. A recent meta-analysis on the influence of PAP therapy on CRP levels in OSAS concluded that at least 3 mo of treatment is required to significantly decrease levels indicating that the inflammatory process is still active through this period[75]. CRP levels further declined after 6 mo of PAP treatment. Furthermore, Xie *et al*[76] also showed a significant decrease on CRP levels, with better benefits with therapy duration of $\geqslant$ 3 mo and more adequate compliance ($\geqslant$ 4 h/night). A previous meta-analysis showed PAP therapy to significantly reduce CRP

levels, by 0.11 mg/dL, or 17.8%[77]. In another study[65] we observed that the division of the patients into a good and a poor PAP compliance group affected CRP evolution, with the good PAP compliance group to show exclusively a statistically significant decrease after 6 mo therapy. Although CRP levels were decreased in the poor compliance group, only a statistically significant trend was observed after 1 year of treatment. Based on that, assuming that PAP use is not adequate according to the generally accepted criteria (use < 4 h per night and < 5 d per week), there is not sufficient influence on OSAS-related cardiovascular sequalae. Once again, as CRP is only a component in the complicated inflammatory process which characterizes OSAS, the evolution of other markers should be considered in order to have final conclusions.

CONCLUSION

Since CVDs are detrimental to human health, medical practitioners seek to predict the development of cardiovascular events before they occur. CRP has been the most studied inflammatory protein to date and a frequently used marker to predict the occurrence of CVDs. Unfortunately, the true correlation between CRP and OSAS is open to controversy, as well as the clinical effect of any reduction. CRP is only one element of the underlying noxius inflammatory process in OSAS. However, there is a shortage of simple, standardized, and cost-effective methods for patient follow-up, and CRP presents these features. In this way, CRP might be valuable along with all other parameters used for the follow-up of patients with OSAS in PAP clinics. Further research is required to define those OSAS patients who will have a considerable reduction with treatment, as well as to understand the significance of the interaction between cardiovascular risk factor and CRP reduction in patients with OSAS.

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REVIEW

Role of the Wnt/β-catenin pathway in gastric cancer: An in-depth literature review

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Abstract

Gastric cancer remains one of the most common cancers worldwide and one of the leading cause for cancer-related deaths. Gastric adenocarcinoma is a multifactorial disease that is genetically, cytologically and architecturally more heterogeneous than other gastrointestinal carcinomas. The aberrant activation of the Wnt/β-catenin signaling pathway is involved in the development and progression of a significant proportion of gastric cancer cases. This review focuses on the participation of the Wnt/β-catenin pathway in gastric cancer by offering an analysis of the relevant literature published in this field. Indeed, it is discussed the role of key factors in Wnt/β-catenin signaling and their downstream effectors regulating processes involved in tumor initiation, tumor growth, metastasis and resistance to therapy. Available data indicate that constitutive Wnt signalling resulting from *Helicobacter pylori* infection and inactivation of Wnt inhibitors (mainly by inactivating mutations and promoter hypermethylation) play an important role in gastric cancer. Moreover, a number of recent studies confirmed *CTNNB1* and *APC* as driver genes in gastric cancer. The identification of specific membrane, intracellular, and extracellular components of the Wnt pathway has revealed potential targets for gastric cancer therapy. High-throughput "omics" approaches will help in the search for Wnt pathway antagonist in the near future.

Key words: Gastric cancer; Wnt; β-catenin; Oncogene; Tumor suppressor; Epigenetics; *Helicobacter pylori*; Adenomatous polyposis coli

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Core tip: Available data indicate that Wnt signaling substantially impacts gastric tumorigenesis, prognosis, and resistance to therapy. Loss of Wnt signaling inhibitors (such as *APC*) by promoter hypermethylation, allelic loss or other mechanisms appear to be particularly important in activating Wnt/β-catenin signaling. Gastric cancer is an entity characterized by its heterogeneity in various aspects, and much remains to be learned about the molecular aspects that determine the process of cancer development in this organ. The role that plays the canonical Wnt pathway in gastric carcinogenesis is reviewed.

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INTRODUCTION

Gastric cancer incidence and mortality have been declining over the past 50 years in most Western countries, but remains the fifth most common cancer and the third leading cause of cancer-related death in both sexes worldwide after lung and breast cancer (723000 deaths, 8.8% of the total in 2012)[1,2]. Gastric cancer incidence varies substantially worldwide, being the highest (more than two-thirds) observed in East Asia, Eastern Europe, and the Andean region of South America, while North America, Northern Europe and North and East Africa show the lowest recorded rates[1]. More than 70% of cases in 2012 (677000 cases) occur in developing countries, and half of worldwide cases occur in Eastern Asia (mainly in China)[3]. The highest estimated mortality rates are in Eastern Asia (24 per 100000 in men, 9.8 per 100000 in women), the lowest in Northern America (2.8 and 1.5, respectively)[3]. Latin American countries and nations from Central and Eastern Europe display some of the highest mortality rates in both sexes worldwide[3].

Diagnosis is usually made after the disease reaches an advanced stage because early gastric cancer produces few symptoms. Therefore, most gastric cancer patients are diagnosed in advanced-stage disease with a poor prognosis. The main treatment option is the gastrectomy combined with chemotherapy and radiation therapy protocols. The poor understanding of the pathogenic mechanisms of gastric cancer and etiological factors, and the lack of effective treatment are reflected in the late diagnosis and high mortality of this disease.

Gastric cancer is a multifactorial disease resulting from the interaction between genetic and environmental factors at the stomach mucosa level. These complex interplays can promote gastric cancer development as result of the deregulation of a number of potentially cell oncogenic signaling pathways, leading to acquisition of malignant phenotypes, including increased cell proliferation, evasion of apoptosis and enhanced invasiveness[4].

The most common histologic variant of gastric adenocarcinoma is the intestinal type. A multistep cascade model for the development of intestinal-type gastric adenocarcinoma consists of a progression from chronic superficial (non atrophic) gastritis, to chronic atrophic gastritis to intestinal metaplasia, followed by dysplasia and finally, gastric adenocarcinoma[5]. This model assumes the sequence of precancerous lesions as a dynamic process from an initial superficial inflammation caused by *Helicobacter pylori* (*H. pylori*) infection to a fully malignant neoplasm of the stomach. Thus, the chronic infection of the gastric mucosa by the *H. pylori*, which colonize approximately 50% of the human population, is considered the strongest known risk factor for the development of gastric cancer[6,7].

Ooi *et al*[8] identified three signaling pathways (nuclear factor-κB, Wnt/β-catenin, and proliferation/stem cell) that were deregulated in more than 70% of the patients diagnosed with gastric cancer, resulting in increased inflammatory cytokine production, abnormal apoptosis, undesirable epithelial cell proliferation/differentiation, and epithelial cell transformation. However, a complete knowledge of the relative importance, temporal activation and crosstalking between these signaling pathways is not well understood yet. Particularly, activation of the Wnt/β-catenin signaling is found in about 30% to 50% of gastric cancer tissues and in many kinds of gastric cancer cell lines[8-10]. In this review, the involvement of the Wnt/β-catenin signaling pathway and its biological significance in gastric carcinogenesis will be discussed.

CANONICAL WNT PATHWAY

The Wnt signaling pathways, named for its ligands Drosophila wingless (wg) and the mouse homolog Int-1 (Wnt-1)[11], are a group of signal transduction pathways which play a fundamental role in the cell fate specification during early embryonic development, proliferation, body axis patterning, survival, apoptosis, and in tissue homeostasis in adults[12].

Wnt signaling pathways can be activated upon binding of Wnt ligands (19 known members in humans) to their cell surface receptor complex, which includes 10 different members of the Frizzled (Fz) family of atypical heptahelical G-protein-coupled receptors[13-15]. Moreover, selective recruitment and participation of other single-pass transmembrane proteins function as co-receptors influencing the Wnt signaling, such as lipoprotein receptor-related protein (LRP)5/6, receptor-like tyrosine kinase (Ryk) and receptor tyrosine kinase-like orphan receptor (ROR)-1/2[16-18].

Up to date, the three best characterized Wnt signaling pathways are the canonical Wnt pathway or β-catenin dependent which involves the stabilization of the proto-oncogene β-catenin (armadillo in Drosophila), the planar cell polarity (PCP) pathway, involved in ciliogenesis, and the Wnt/Ca^{2+} pathway, which stimulates the intracellular release of Ca^{2+} and activates Ca^{2+}-dependent mediators involved in the control of cell movement and behavior[19-21]. These two last pathways are collectively named as "non-canonical" or "β-catenin-independent" pathways, that also include a steadily increasing number of complex signaling routes: Wnt/cAMP, Wnt/Ror, Wnt/Rap, Wnt/Rac and Wnt/Rho pathways[19,22].

The Wnt/β-catenin intracellular signaling pathway is highly conserved among metazoan species[20,23]. β-catenin is a multifunctional protein that was found as

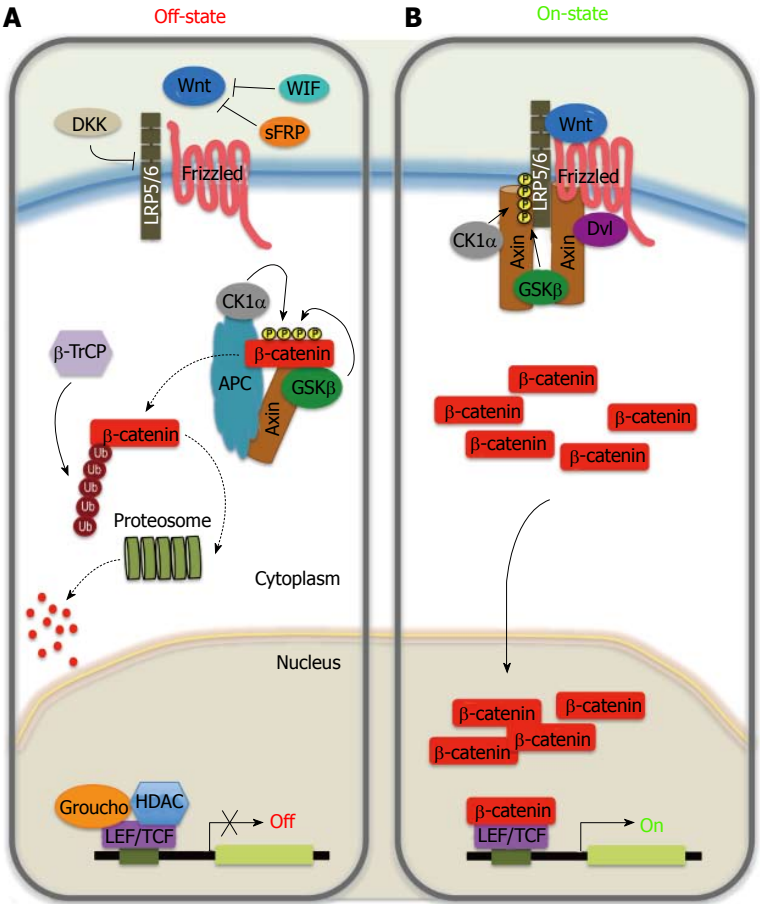

Figure 1 Wnt/β-catenin signalling pathway. A: In the absence of Wnt ligands, cytoplasmic β-catenin binds to the "destruction" complex composed by Axin, adenomatous polyposis coli (APC), the glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α). β-catenin is phosphorylated by the kinases of the complex on a set of conserved Ser and Thr residues in its amino terminus. The phosphorylated form of β-catenin is recognized by an E3 ubiquitin ligase (β-TrCP) and then targeted to proteosomal degradation, resulting in low cytosolic levels. In the absence of nuclear β-catenin, T cell-specific factors (TCF)/lymphoid enhancer-binding factor (LEF) proteins repress target genes expression through a direct association with transcriptional inhibitors of the Groucho family and histone deacetylases (HDACs); B: In the presence of Wnt ligand, secreted Wnt binds to their receptor complex, consisting of Frizzled and one member of the low-density lipoprotein receptor family (LRP5/6). This binding disrupts the "destruction complex" by recruiting Dishevelled (Dvl) to the cytoplasmic domain of Frizzled, which induces the delocalization of cytoplasmic Axin to the cytoplasmic tail of LRP and phosphorylation by CK1α and GSK3β of the latter. The disruption of the "destruction" complex prevents phosphorylation/degradation of β-catenin, leading to accumulation of β-catenin in the cytoplasm. Active ß-catenin translocates to the nucleus, where it acts as a transcriptional co-activator with TCFLEF to activate Wnt-responsive target genes. Extracellular Wnt signaling can be inhibited by binding of members of the secreted frizzled related protein (sFRP) and Wnt inhibitory factor (WIF) families to Wnt ligands, or by the interaction of soluble Dickkopf (DKK) with LRP.

a E-cadherin-binding protein involved in the regulation of cell to cell adhesion, and as a transcriptional regulator in the Wnt signaling pathway[17]. It is assumed that the disruption of cadherin/catenin complexes is involved in the malignant progression of epithelial tumors.

In the absence of Wnt ligands, cytoplasmic β-catenin is bound by the destruction complex composed of the scaffolding proteins Axin, adenomatous polyposis coli (APC), and the serine/threonine kinases, Casein kinase 1 alpha (CK1α) and glycogen synthase kinase 3β (GSK3β)[24]. Phosphorylated β-catenin is targeted to proteosomal degradation after recognition by the E3 ubiquitin ligase β-TrCP resulting in low cytosolic levels of β-catenin[25]. As a consequence, the transcription factor T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) binds to transcriptional inhibitors of the Groucho family, that recruit histone deacetylase to mediate transcriptional repression through chromatin compaction[26,27].

In contrast, when the secreted Wnt ligands bind to their Fz/LRP receptor complex, the dissociation of the destruction complex occurs by recruiting Dishevelled (Dvl) to the cytoplasmic domain of Fz. This association results in LRP phosphorylation and the delocalization of cytoplasmic Axin by its recruitment to the receptors. These events prevent phosphorylation and subsequent degradation of β-catenin, which accumulates in the cytoplasma and translocates to the nucleus. For its nuclear action, β-catenin behaves as transcriptional co-regulator by interacting with TCF/LEF complex, displaces Groucho, and activates Wnt target gene expression, such as *CCND1* (encoding cyclin D1 protein), gastrin and *c-myc*[27,28].

Even in the presence of Wnt ligand, the Wnt/β-catenin pathway can be inhibited through the binding of soluble Dickkopf (Dkk1-4) to LRP5/6, or by Wnt-inhibitory factor (WIF-1) and the family of secreted Fzd receptor proteins (sFRP1-5) that can interact with Wnt ligands preventing them from binding to receptors[29-31].

Activity of the canonical Wnt signaling pathway is dependent on controlling the accumulation of β-catenin in the cytoplasmic and nuclear compartments (Figure 1)[20,32,33]. β-catenin is normally constitutively transcribed and translated. It has been suggested that β-catenin is regulated by its cytoplasmic retention by both cadherins and Axin that sequester β-catenin in the plasma membrane and cytoplasm, respectively[34]. Upon receptor activation a signal-induced block in proteolysis leads to rapid rise in cytosolic β-catenin protein levels that subsequently also accumulates in the nucleus. Moreover, β-catenin does not contain any recognizable nuclear localization signal or export signal, and its nuclear entry is independent of classic import factors. Finally, it has been proposed that the nuclear build-up of β-catenin results from a combination of anchoring for β-catenin in the nucleus by nuclear proteins (TCF, LEF-1, Pygopus, and BCL9) and the nuclear export, in which Axin, APC,

Table 1 Components of Wnt/β-catenin signaling deregulated in gastric cancer

Upregulation or function increase in gastric cancer	
Wnt-1, Wnt-2, Wnt-2B, Wnt-5a, Wnt-6, Wnt-10A[47,69-75,232]	
Fzd-3[78]	
CTNNB1[9,88-93,96,97]	
LRP6[228]	
TCF7L2[98-100]	
PPN[79]	
CDH17[80]	
EZH2[81]	
HMGA1, HMGA2[210,211]	
YY1[86]	
TC1 (C8orf4)[201,202]	
miR-17-92[161]	
mir-10a[168]	
has-miR-335[166]	
hsa-miR-375[163]	
Downregulation or function inhibition in gastric cancer	
Downregulation by hypermethylation	Inactivation by miRNAs
APC[145,146]	*APC*[175]
sFRP1, sFRP2, sFRP4, sFRP5[78,96,149]	*AXIN2*[159]
WIF-1[144,149]	*EZF1*[78]
Dkk-1, Dkk-2, Dkk-3[59,144,148,149]	*HIPK1*[161]
NKD1[149]	*HSulf-1*[168]
Sox10, Sox17[106,107,112,149]	*RUNX2*[159]
HSulf-1[108]	Inactivation by phosphorylation
RUNX3[147]	*GSK3β*[105]
PRDM5[152,153]	Inactivation by overexpression of EZH2
RASSF10[154]	*CXXC4*[81]
OSR1[111,112]	Other or undefined mechanisms
Inactivation by protein mislocalization	*Sox7*[113]
RUNX3[147]	*RACK1*[109]
Inactivation by *H. pylori* virulence factor	*ZNRF3*[110]
RNF138[226,227]	*CHD8*[131]
Deletion or mutation	*PLA2G2A*[205]
APC[92,104,133-137]	*has-miR-148a*[165]
GSK3β[104]	*miR-193b*[163]
β-TrCP[142]	*miR-200a*[176,177]
AXIN1, AXIN2[90,91]	

APC: Adenomatous polyposis coli; AXIN: Axis inhibition protein; β-TrCP: β-transducin repeats-containing protein; CDH: Cadherin; CHD: Chromodomain helicase DNA binding protein; CTNNB1: Cadherin-associated protein (ß-catenin); CXXC: CXXC finger protein; Dkk: Dickkopf; Fzd: Frizzled; EZF: Endothelial zinc finger; EZH2: Histone-lysine N-methyltransferase; GSK3β: Glycogen synthase kinase-3β; HIPK: Homeodomain interacting protein kinase; HMGA2: High-mobility group AT-hook; HSulf: Human endosulfatase; LRP: Low density lipoprotein receptor-related protein; NKD: Naked cuticle homolog; OSR: Odd-skipped-related; PLA2GA2: Phospholipase A2; PPN: Palmitoyltransferase porcupine; PRDM: PR domain containing; RACK: Receptor for activated C kinase; RAFFS: Ras association domain family; RNF: Ring finger protein; RUNX: Runt-related transcription factor 3; sFRP: Secreted frizzled-related protein; SOX: Sry-related HMG box; TCF7L2: Transcription factor 7-like 2; WIF: Wnt inhibitory factor; Wnt: Wingless-type; YY1: Ying Yang 1; ZNRF: Zinc and ring finger.

and RanBP3 have been implicated[35-41].

WNT/β-CATENIN PATHWAY IN GASTRIC CARCINOGENESIS

Homeostasis of the gastrointestinal epithelium is strongly dependent on the balance existing between cell proliferation, cell cycle arrest, and cell migration. The canonical Wnt signaling pathway, in addition to its role in early embryogenesis, plays a crucial role in regulating proliferation, stem cell maintenance and homeostasis in normal gastric mucosa[42-44]. On the other hand, it is recognized that dysregulation of the Wnt pathway plays a critical role in the development of human cancer, with more than 30% of gastric cancer in which activated Wnt/β-catenin signaling can be found. Furthermore, the essential role of Wnt/β-catenin signaling in the self-renewal of gastric cancer stem cells (GCSC) has been demonstrated[45-47]. Several components of the Wnt pathway (Figure 1) may be overexpressed or their function increased by other mechanisms in gastric cancer (Table 1). Moreover, the loss of Wnt inhibitors may play an important role in gastric carcinogenesis (Table 1).

Large-scale sequencing studies conducted in many tumor types have identified co-occurrence of mutations in positive and negative regulators of the Wnt pathway. However, the prevalence of these mutations seems to be cell-type specific[48,49]. Moreover, a number of recently published studies exploring gene mutations in gastric cancer using high-throughput methodologies (Next-generation sequencing and genotyping array) have confirmed *CTNNB1* (the gene that encodes β-catenin protein) and *APC* as driver genes. Although with variations in prevalence, these studies revealed somatic mutations in both genes that might be relevant in gastric carcinogenesis[50-55].

In addition to multiple genetic alterations, the initiation and progression of gastric cancer are also associated to epigenetic changes[56,57]. Histone modification and promoter CpG methylation alter cancer-related gene expression and are frequently involved in carcinogenesis[58]. So far, downregulation of Wnt antagonist genes associated to promoter hypermethylation have been identified in a variety of malignancies, such as renal, bladder, lung, breast, colorectal, gastric and neuroblastoma[59-65].

Furthermore, the Wnt/β-catenin pathway genes are found among those affected by dysregulation of microRNAs (miRNAs) in many kinds of cancers, and particularly, expression profiling has shown that certain miRNAs are associated with gastric cancer development, progression and response to therapy[66-68].

Gain of Wnt activator function in gastric cancer

Members of the Wnt family protein, such as Wnt-1, Wnt-2 and Wnt-2B have been found upregulated in gastric cancer[47,69-71]. The overexpression of *Wnt-2* was associated with cytoplasmic/nuclear β-catenin accumulation both in intestinal- and diffuse-type gastric carcinoma, and positively associated with increased metastatic potential[70]. Furthermore, the upregulation of Wnt-1 ligand was found playing an important role either in cellular proliferation of GCSC and in advanced gastric cancer[47,71]. In this regard, in transgenic mouse models, Oshima *et al* observed that Wnt-1 expression together with activation of PGE_2 pathway result in invasive gastric

adenocarcinoma formation by 1 year[72].

Wnt-5a, which is upregulated in gastric cancer regardless of their histological phenotype, has an increased expression in advanced stages of gastric cancer and it is correlated with poor prognosis[73,74]. Furthermore, Yuan *et al*[75] demonstrated that caveolin-1 (Cav1) and Wnt-6 positively cooperate in promoting chemoresistance of gastric cancer cells to DNA-damaging anthracycline drugs through activation of the canonical Wnt/β-catenin pathway. Caveolin is an integrated plasma membrane protein and is involved in lipid transport, membrane trafficking, gene regulation and signal transduction[76]. Certain cancer cells that express caveolins are more aggressive and metastatic, as well as drug-resistant[77].

The upregulation of *Fzd-3*, Wnt palmitoyltransferase porcupine (*PPN*), and *CDH17* has been found to be critical for cell proliferation and activation of the Wnt/β-catenin signaling pathway in gastric cancer[78-80]. Moreover, Lu *et al*[81] have provided evidence indicating that EZH2 (histone methyl-transferase, enhancer of zeste homolog 2) activates Wnt signaling in gastric cancer mainly by downregulating the expression of CXXC4 (CXXC finger protein 4) without involving DNA methylation. It was also confirmed that overexpression of *CXXC4* disrupts the association of Dvl with Axin-GSK3β by directly interacting with Dvl, thus functioning as a tumor suppressor[81].

Upregulation of Actin-binding protein anillin (ANLN), a protein involved in the cytokinesis and known to be dysregulated in many cancers, was found responding to the activity of Wnt/β-catenin pathway in gastric cancer[82]. Moreover, elevated expression of *ANLN* was identified as a molecular predictor of intestinal and proliferative type gastric cancer[82]. Similarly, cyclin-dependent kinase 8 expression and the delocalization of β-catenin expression have shown a significant positive correlation with carcinogenesis and tumor progression, especially lymph node metastasis[83].

On the other hand, the ubiquitously distributed transcription factor Yin Yang 1 (YY1) can act either as a tumor suppressor gene or as an oncogene depending on the type of tumor. This dual behavior might be determined by cell context, oncogenic stimulation or the regulation of its upstream pathways[84,85]. YY1 was found to promote the Wnt signaling pathway in gastric cancer, probably by suppressing Wnt antagonists[86]. *YY1* expression is involved in the carcinogenesis of diffuse-type gastric carcinoma, and it was correlated with poor prognosis in patients with early stage gastric cancer[86].

It has been suggested the existence of an interplay between the Wnt signaling cascade and Notch1 signaling pathway in gastric cancer cells, in which the aberrant activation of Wnt/β-catenin signaling overcomes the pro-apoptotic role of Notch in gastric cancer cells[87]. Furthermore, Li *et al*[87] observed that when the two signaling pathways were simultaneously activated, there was a combined effect of promoting the proliferation of gastric cancer BGC-823 cells by upregulating the expression of c-Myc, cyclin D1, cyclin E and CDK2.

Genomic alterations: In contrast to the colorectal cancer, mutations driving gastric cancer are much less defined. Mutations in *CTNNB1* have been frequently detected (approximately 30%) in intestinal- and diffuse-type gastric carcinomas displaying nuclear accumulation of β-catenin[9,88-92]. These mutations occur mainly in exon 3, that encodes for the GSK3β phosphorylation consensus region of the β-catenin gene, resulting in mutants refractory to regulation by the destruction complex, and thus in accumulation of this protein and constitutive activation of the Wnt pathway. In contrast, there are studies examining the incidence of *CTNNB1* mutations that report their infrequency, either in intestinal and diffuse-type gastric cancer[93-96].

Wang *et al*[97] performed a genetic association study (944 patients and 848 cancer-free controls) to analyze the correlation between five tagged single nucleotide polymorphisms (SNP) spanning the *CTNNB1* gene (rs4135385, rs1798808, rs1880481, rs11564465 and rs2293303) and gastric cancer risk and prognosis. Their results showed that the SNPs rs1880481, rs4135385, rs11564475 and rs2293303 were significantly associated with gastric cancer susceptibility. In addition, the rs4135385 AG/AA genotypes were associated with a 0.74-fold reduced adjusted hazard ratio for favorable overall 5-year survival of non-cardia gastric cancer.

In the nucleus, free β-catenin binds members of the TCF/LEF family of transcription factors, including TCF7L2 (TCF4), thereby modulating gene transcription[27,28]. Somatic frameshift mutations of *TCF7L2* in the repeat sequence A9 have been frequently detected in gastric cancers with microsatellite instability (MSI)[98,99]. On the contrary, no MSI mutations in *TCF7L2* were detected in Japanese patients with sporadic gastric tumors[100]. In this sense, about 15% to 50% of gastric carcinomas are categorized into MSI–positive cancers[101].

Considering post-transcriptional modifications, the splicing pattern of numerous genes is altered as cells progress in the tumorigenesis process. It is hypothesized that alternative spliced exons can originate functional protein–protein interactions and establish tissue-specific proteome signatures[102]. The participation of Wnt/β-catenin pathway in alternative splicing in colorectal, lung and gastric cancer was identified by a mechanism involving the up-regulation of the serine/threonine-protein kinase SRPK1 and the splicing factor SRSF1[103]. Furthermore, identification of TCF4 binding sites both in the promoter regions of *SRPK1* and *SRSF1* makes these two genes apparent Wnt-targets.

Loss of Wnt repressor function in gastric cancer

Several studies have demonstrated that loss of Wnt inhibitors may play a major role in gastric cancer (Table 1). In mouse models, deletion of *APC* or *GSK3*β leads to rapid nuclear β-catenin accumulation and Wnt target gene

expression that result in formation of benign lesions, such as intestinal-type adenomas and fundic gland polyps but not tumorigenesis[104]. These molecular changes seem to be required to drive early carcinogenesis events in the stomach, a hypothesis that is supported by studies showing that GSK3β phosphorylation and inactivation is associated with cancer progression in gastric cancer[105]. Different studies have shown downregulation-in occasion by unspecified mechanisms-of various negative regulators of the Wnt/β-catenin signaling involved in proliferation and invasion in gastric cancer, including *sFRP*, *Sox7*, *Sox10*, *Sox17*, *HSulf-1*, *RACK1*, *ZNRF3* and *OSR1*[78,106-113].

Some Wnt-target genes, such as *Dkk-1*, Axin, Nemo kinase, *etc.*, have shown to cause inhibition of Wnt signaling itself[114,115]. These Wnt-antagonists are initially upregulated in response to Wnt signaling activation, initiating a negative feedback loop[116]. However, in certain tumor cells, the epigenetic silencing of Dkk-1 by promoter hypermethylation or loss of heterozygosity, produces an imbalance of Wnt/Dkk negative feedback, therefore contributing to persistent activation of Wnt/β-catenin signaling[117,118]. Moreover, recently it was reported that Polycomb group protein BMI1, which is transcriptionally regulated by the Wnt-target gene c-Myc, act as an activator of the Wnt pathway by repressing Dkk protein family (particularly Dkk-1) as part of positive feedback loop[119].

Conversely, as despite Dkk-1 being a Wnt pathway inhibitor, this protein has been found upregulated in several human cancers, such as lung, esophageal, breast, myeloma multiple and gastric cancer[120-123], indicating that Dkk-1 could has a potential oncogenic role in these tumors rather than acting as a tumor suppressor by antagonizing Wnt signaling. That elevation of Dkk-1 expression is likely to be caused by some epigenetic alterations including the loss of promoter methylation of the *Dkk-1* gene[123,124]. Moreover, the oncogenic activities of Dkk-1 could be mediated, at least in part, by non-canonical Jun-mediated Wnt pathways activation.

RUNX3 suppresses the canonical Wnt signaling pathway by forming a ternary complex of RUNX3, TCF4 and β-catenin, that inhibits the binding of the complex to the Wnt target gene promoter, thereby suppressing Wnt signaling[125]. This transcription factor has been originally identified as a tumor suppressor, and RUNX3 loss is seen as an early event in gastric cancer development due to aberrant Wnt signaling, causing spontaneous epithelial-mesenchymal transition (EMT) and producing a tumorigenic stem cell-like subpopulation[126-128]. Furthermore, recent evidence suggests that RUNX3 can either suppress or activate Wnt signaling activity in gastric cancer by binding to the TCF4/β-catenin complex, depending of (or regulated by) cell context-dependent mechanisms[129]. Similarly, sFRP1, which is classically considered as an inhibitor of Wnt/β-catenin signaling, has been observed playing in promoting gastric tumourigenesis. This double-edged role seems depend on the strong link between sFRP1 expression and TGFβ signalling activation. In gastric cancer cells high sFRP1 levels are correlated with the activation of TGFβ signaling[130].

A recent study demonstrated that loss of *CHD8* expression, commonly observed in gastric cancer, represents an indicator for the biological aggressiveness of gastric cancer[131]. Nishiyama *et al*[132] suggested that CHD8 (chromodomain helicase DNA-binding) negatively regulates β-catenin function by recruiting histone H1 to the promoters of Wnt target genes, thereby functioning as a tumor suppressor.

Genomic alterations: *APC* mutations have been detected in both precancerous and malignant gastric lesions with up to 25% of gastric adenomas exhibiting somatic mutations in this gene[133,134]. Moreover, deletions of *APC* at the chromosome locus 5q21-22 have also been found in 20%-60% of gastric cancer[135-137]. Ogasawara *et al*[92] observed that gastric tumors had mutations of the *APC* gene comparable to the frequency of *CTNNB1* mutation. They suggested that such *APC* mutations explain the abnormal β-catenin localization and accumulation that could cause activation of Wnt/β-catenin signaling in some cases. Furthermore, in a murine model heterozygous for E-cadherin gene deletion, loss of heterozygosity of *APC* synergistically enhanced gastric tumor initiation[138]. The fact that *APC* gene deletions were mainly observed in advanced tumors suggests that it might occur in the progression but not in the initiation of gastric cancers[137]. In addition, loss of the mesenchymal transcription factor *Foxl1* leads to an earlier tumor initiation due to accelerated loss of heterozygosity at the *APC* locus in colon and stomach[139].

Axin1 and Axin2 proteins negatively regulate the Wnt signaling pathway based in their roles as scaffolding proteins involved in the assembly of the β-catenin destruction complex. Therefore, mutations in their genes have been proposed as critical defects in some cancers[140]. Moreover, *Axin2* is a transcriptional target of the LEF/TCF-β-catenin transcription factor complex[141]. A highly mutable G mononucleotide repeats present in exon 7 of *Axin2* frequently has a frameshift mutation (1 bp deletion) in gastric cancer with MSI and nuclear β-catenin expression[90,98]. *Pan et al*[90] also identified in their study in gastric cancer five SNPs (334 C>T, 874 C>T, 1396 G>A, 1690 C>T and 1942 T>G) and frameshift mutations in *Axin1*.

Missense mutations in *β-TrCP*, a component of the ubiquitin ligase complex targeting β-catenin for proteasomal degradation, also has been suggested to contribute to the development of gastric cancer through β-catenin stabilization[142]. Finally, somatic mutations in Wilms' tumor gene on the X chromosome (*WTX*), which is also considered a negative regulator of Wnt/β-catenin signaling, have been only detected in

colorectal cancer, but not in gastric carcinomas[143].

Epigenetic modifications: Hypermethylation of Wnt-antagonist and tumor suppressor genes may be one of the key mechanisms for the translocation of β-catenin to nucleus and subsequent aberrant activation of canonical Wnt/β-catenin pathway involved in the pathogenesis of gastric cancer[144,145]. Indeed, *APC* is one of the genes commonly hypermethylated in gastric cancer[145,146]. Moreover, hypermethylation of *APC* promoter contributing to moderate activation of Wnt signaling was associated to the development and progression of gastric adenomas, which are considered as premalignant lesions of gastric adenocarcinoma[145]. This process seems to be accelerated by infection with *H. pylori*[145].

Several other tumor suppressor genes that function through modulating Wnt/β-catenin signaling were found silenced in gastric cancer, such as members of the *sFRP* and *Dkk* gene families. CpG methylation-depending silencing of *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5*, *HSulf-1*, *WIF-1*, *RUNX3* as well as, *Dkk-1*, *Dkk-2* and *Dkk-3* has been frequently observed among gastric cancer cells lines and primary specimens[59,96,108,144,147,148]. In addition, Yu *et al*[148] showed by multivariate analysis that *Dkk-3* methylation was associated significantly and independently with poor disease survival in gastric cancer, but not in colorectal cancer. Recently, DNA methylation status in 49 gastric cancer samples was analyzed by a bead array with 485512 probes[149]. This study revealed that the Wnt pathway was potentially activated by aberrant methylation of its negative regulators, such as *Dkk-3*, *NKD1*, *Sox17*, *WIF-1* and *sFRP1*. Moreover, DNA methylation and extensive mutation analyses in gastric cancers revealed an association between the CpG island methylator phenotype and oncogenic mutations in *CTNNB1*, *ERBB2*, *KRAS* and *PIK3CA*[150].

Downregulation of *Sox10* by promoter methylation has also been detected in gastric cancer tissues[112]. Sox (SRY-related-HMG-box) familiy members are transcription factors that regulate canonical Wnt signaling through different mechanisms, including protein-protein interactions and binding to Wnt-target gene promoters[151]. Sox10 is an important DNA binding transcription factor that recruits β-catenin to repress Wnt/β-catenin signaling in cancer progression[112].

PRDM5, an epigenetic modifier gene, was found inactivated by promoter methylation in 76% of gastric cancer[152,153]. While knockdown of *PRDM5* expression induced cell proliferation, ectopic *PRDM5* expression significantly inhibited tumor cell clonogenicity, accompanied by the downregulation of TCF/β-catenin-dependent transcription of *CDK4*, *TWIST1* and *MDM2* oncogenes[153].

Moreover, silencing of *RASSF10*, a member of the Ras association domain family proteins that are considered as tumor suppressor genes, was recently found associated with promoter hypermethylation in gastric cancer cell lines[154]. This study also demonstrated that *RASSF10* over-expression inhibited the Wnt/β-catenin signaling pathway in gastric cancer cells.

Odd-skipped related 1 (*OSR1*), a gene encoding a zinc-finger transcription factor, was found preferentially methylated in gastric cancer by genome-wide methylation screening[111]. The tumor suppressive activity of *OSR1* in gastric cancer is in part due to inhibition on the expression of *LRP6*, *CTNNB1*, *TCF-1*, *LEF1*, and *Axin2* in the Wnt/β-catenin signaling pathway. In this work, *OSR1* methylation was also considered as an independent predictor of poor survival of patients at early stage of gastric cancer[111].

Role of microRNAs in Wnt/β-catenin signaling in the gastric cancer

MiRNAs are short noncoding RNA molecules of 19-25 nt that regulate gene expression at the post-translational level leading to mRNA degradation or the inhibition of translation[155]. MicroRNAs are involved in the regulation of different biological processes, including apoptosis, proliferation, metabolism, and as modulators of development they can promote either self-renewal or differentiation in stem cells to determine their fates[155,156]. Moreover, miRNA expression profiling in many kind of cancers has shown that certain miRNAs can function as oncogenes or tumor suppressors, and their misexpression and dysregulation contribute to the tumorigenic process[157].

A miRNA expression profile in normal gastric tissues have been described[158]. Moreover, an increasing number of studies have shown dysregulation of miRNAs in gastric cancer tissues and their potential use as biomarker for this disease[66-68]. Although with some controversies, these studies have resulted in the identification of hundreds of differentially expressed miRNAs, some of them involved in regulation of the Wnt/β-catenin signaling[159,160].

Wu *et al*[161] reported that members of the miR-17-92 cluster (miR-19b, miR-20a and miR-92a) act regulating the self-renewal of GCSC by targeting *E2F1* and *HIPK1*, that are known to suppress Wnt/β-catenin signaling. As a consequence, miR-17-92 subsequently activates Wnt/β-catenin signaling[161]. Other genes involved in Wnt pathway were revealed as potential target of miR-30a (*Runx2*, *CDH1*, *Wnt5A*) and miR-103 (*Axin2*) in expression profiling studies in gastric cancer[160]. Moreover, hsa-miR-375 upregulation and hsa-miR-142-5p downregulation have been indicated as predictor for recurrence risk in gastric cancer patients following surgical resection, probably by regulating several Wnt/β-catenin target genes[162].

In Chinese patients with gastric cancer other miRNA genes that regulate Wnt/β-catenin target genes, such as miR-144, hsa - miR - 148a and miR-193b were found downregulated[163-165]. While miR-193b can inhibit tumor invasion and metastasis[163], hsa-miR-148a has been suggested as biomarker for early gastric cancer[165].

Moreover, also in Chinese patients, the survival analyses results showed that patients with high expression levels of hsa-miR-335 had reduced overall survival rate and high recurrence risk of gastric cancer[166]. Among hsa-miR-335 targeted genes is *Fzd-3*, a member of the FZD receptor family, upregulated in several cancers[167].

Metastatic potential of gastric cancer cells have been associated with changes in the expression profile of miRNAs involved in regulating Wnt signaling pathway. MiR-10a upregulation and miR-516a-3p decreased expression were proven to play a pivotal role in tumor invasion and metastasis from primary gastric cancer to lymph nodes or peritoneum, respectively[168,169]. Using proteomics approaches, Takey *et al*[169] showed that miR-516a-3p targets extracellular sulfatase 1 (HSulf-1). HSulf-1 removes 6-O sulfates from heparan sulfate proteoglycans chains to liberate Wnt ligands, and thus, enable them for binding to Frizzled receptors and subsequently activate the Wnt canonical pathway[170]. However, as it was mentioned above, loss or downregulation of *HSulf-1* is associated with carcinogenesis in several tissues.

Tang *et al*[171] identified a novel role for GSK3β in the regulation of miRNAs biogenesis in gastric cancer cells. They showed that GSK3β inhibits the expression of miR-96, miR-182 and miR-183 through the β-catenin/TCF/LEF-1 pathway. GSK3β protein levels are decreased in human gastric cancer tissue compared with surrounding normal gastric tissue, coinciding with increased levels of β-catenin and miR-96, miR-182, miR-183. In addition, overexpression of β-catenin enhances the expression of miR-96, miR-182 and miR-183 in human gastric cancer cells[171].

A recent study identified the overexpression of miR-200b and miR-22 as the mechanism related to the anti-tumor effects of Diallyl disulfide in gastric cancer cells[172]. Furthermore, *Wnt-1* was identified as a target of both miR-200b and miR-22. Moreover, *MAPRE1*, which acts as an activator of the Wnt/β-catenin pathway may be a direct and functional target of miRNA-10b in gastric cancers[173,174].

MiR-27 has been identified as modulator of Wnt signaling by interacting with *APC* and also promoting human gastric cancer cell metastasis by inducing EMT[175]. Finally, it was recently revealed that the upregulation of miR-200a increases E-cadherin and suppresses the Wnt/β-catenin pathway by targeting *CTNNB1*, *ZEB1* and *ZEB2* in gastric cancer[176,177].

Wnt/β-catenin signaling in the gastric cancer stem cells

Cancer stem cells (CSCs) are a group of heterogeneous cells representing a fraction of certain tumors that share many properties with somatic stem cells, such as the capacity for self-renewal and multipotent differentiation potential[178]. CSCs are involved in tumor development, and form the basis for tumor proliferation and metastasis[179-181]. Researchers proved the existence of CSCs in many solid tumors, including in various gastrointestinal neoplasms such as colon, gastric pancreas, liver and gastroesophageal tumors[181-184]. Moreover, CSCs are thought to be responsible for tumor recurrence, which is one of the biggest problems in cancer therapy.

Although the mechanism by which CSCs develop remains unclear several studies have investigated the role of dysregulation of the Wnt/β-catenin, transformation growth factor-β (TGF-β) and Hedgehog pathways in generation of CSC[185-187]. Abnormal activation of Wnt/β-catenin signaling pathway strongly correlates with tumorigenesis and progression through maintaining CSCs[188]. In this regard, Zhu *et al*[189] identified that Dkk-1 secreted by mesenchymal stem cells was able to inhibit proliferation of human carcinoma cell lines through the canonical Wnt signaling pathway. Moreover, knockdown of β-catenin resulted in inhibition of CSC in lung cancer[190].

The development of gastric cancer is closely associated with GCSC. Clinicopathological analysis in gastric cancer has demonstrated that the expression of stem cell markers CD44 and CD133 is highly associated with the degree of malignancy and tumor grading[191-193]. A few studies have explored the role of Wnt/β-catenin signaling in GCSC self-renewal, proliferation and sensibility to treatment.

The squamo-columnar junction (SCJ) region of the normal mouse stomach contains a gland consisting of $CD44^+$ cells, of which a few are quiescent or slow-cycling stem cell-like gastric cells[45]. These $CD44^+$ cells in the SCJ region can be expanded by the cooperative effect of PGE_2-mediated inflammatory signaling and Wnt signaling, suggesting a mechanism to development of gastric tumors in mice[45]. Moreover, Mao *et al*[47] observed that stem-related genes *Wnt-1* and *CD44* are overexpressed in gastric cancer specimens. They found that the proliferation rate of GCSC was dramatically inhibited by knockdown of *Wnt-1*. Also, Salinomycin, an antibiotic thought to suppress Wnt/β-catenin signal transduction can inhibit GCSC[47,194]. Finally, the importance of the Wnt/β-catenin pathway for the self-renewal of CSCs in human gastric cancer have also been suggested by proving that stimulation with Dkk-1 could decrease the self-renewal ability of cancer stem-like cells[46].

Wnt/β-catenin signaling and metastatic potential in gastric cancer cells

A distinctive feature of gastric cancer is early invasiveness into adjacent tissues and the peritoneal cavity. It has been hypothesized that Wnt/β-catenin signaling could contribute to tumor progression by enhancing the proliferation and invasiveness of gastric cancer cells. Furthermore, it is thought that the Wnt/β-catenin pathway is one of the major signaling pathways involved in EMT and therefore, playing a critical role in metastasis[195]. Indeed, disruption of Wnt/β-catenin pathway has shown anti-metastatic activity in gastric cancer cells[196].

In this regard, an important player is Wnt-5a that stimulates cell migration and invasion of gastric cancer cells through regulation of focal adhesion complexes by activating focal adhesion kinase and small GTP-binding protein Rac[74]. Wnt-5a also contributes to gastric cancer progression by inducing expression of laminin γ2, thus increasing metastatic potential[197].

On the other hand, nuclear β-catenin is correlated with an invasive phenotype in intestinal-type tumors[198]. This increase in tumor invasion is likely mediated by the upregulation of Wnt transcriptional targets, such as EphA2 (erythropoietin-producing hepatocellular) and membrane type 3 matrix metalloproteinase (MT3-MMP) genes[199,200]. Furthermore, evidence provided by Kim *et al*[201,202] suggests that TC1 (C8orf4), which is upregulated in high-grade gastric cancers, coordinates the upregulation of Wnt/β-catenin target genes involved in the aggressive biological behavior and poor clinical outcome observed in advanced gastric cancer[202,203]. TC1 enhances the signaling pathway by relieving the antagonistic regulation of Chibby (Cby) on β-catenin-mediated transcription[203]. Cby was first identified as an interactor with β-catenin in humans, which suggested it could be a tumor suppressor gene[204].

Silencing of *PLA2G2A* (phospholipase A2) enhances the invasive ability of gastric cancer cells[205]. Surprisingly, *PLA2G2A*, which is an independent predictor of favorable outcome for patients with gastric cancer[206,207], is also a direct Wnt target gene and it has been found upregulated in early stages of gastric cancer. Ganesan *et al*[205] have proposed that in late-stage tumors, Wnt signaling is still active driving expression of proto-oncogenes; however, *PLA2G2A* expression is decreased possibly by epigenetic inactivation and/or genomic deletions, resulting tumors with a highly aggressive clinical phenotype. Moreover, the overexpression of the Wnt signalling regulator *sFRP1* has been correlated with activation of TGFβ signalling pathway, thereby it may induce cell proliferation, EMT and invasion[130].

HMGA (high mobility group protein) proteins contain DNA binding domains that mediate binding to AT-rich regions of chromatin, having the ability to modulate transcription of their target genes by altering the chromatin structure at the promoter and/or enhancers[208]. Members of HMGA family, *HMGA1* and *HMGA2*, are highly expressed during embryonic development and are linked to malignant transformation and progression in human cancers, including gastric cancer[208,209]. *HMGA1*, is induced by the Wnt/β-catenin pathway and maintains proliferation of gastric cancer cells[210]. Moreover, HMGA2 activates the Wnt/β-catenin pathway and causes EMT in gastric cancer cells by suppressing the expression of *Axin1*, thereby protecting β-catenin from phosphorylation degradation[211]. HMGA2 also promotes β-catenin translocation by increasing expression of *TWIST1*, which can repress E-cadherin expression[211].

Role of infections in enhancing Wnt/β-catenin pathway signaling in gastric cancer

H. pylori virulence factors contributing to gastric cancer development include cytotoxin-associated gene product (cagA), vacuolating cytotoxin A (vacA), blood group antigen binding adhesion 2 (BabA), outer inflammatory protein (oipA) and induced by contact with epithelium (iceA)[212].

Infection with cagA-positive *H. pylori* strains increases the risk of peptic ulcers and gastric carcinoma[213]. CagA protein is translocated by a type Ⅳ secretion system into gastric epithelial cells where forms a physical complex with the Src homology 2 domain-containing tyrosine phosphatase in a phosphorylation dependent manner[214]. These changes trigger a complex set of alterations in signal transduction that affects various cellular processes[215]. Another *H. pylori* constituent linked to the development of gastric cancer is the secreted VacA toxin. VacA can cause a wide assortment of alterations in gastric epithelial cells, including cell vacuolation, gastric epithelial cell autophagy and apoptosis[216,217]. VacA can also interfere with T cell activation, which suppresses local immune response[218].

H. pylori infection could cause cancer tumorigenesis and malignant transformation of host cells through the activation of oncogenic pathways[219]. In this sense, evidence has raised the possibility that Wnt/β-catenin pathway plays a role in the pathogenesis of *H. pylori* infection, including the promotion of pathological changes seen in gastric cancer. Indeed, *in vitro H. pylori* infection of cells promotes an increase in nuclear levels of β-catenin and TCF/LEF transactivation, with upregulation of the β-catenin target gene *cyclin D1* in a CagA-independent manner[220,221].

Among the pleiotropic effects of the vacuolating cytotoxin VacA, Nakayama *et al*[222] reported that this *H. pylori* virulence factor induces the Wnt/β-catenin pathway through the activation of the PI3K/Akt signaling pathway. This triggers the phosphorylation and inhibition of GSK3β, and β-catenin translocation to the nucleus after dissociation of GSK3β/β-catenin complex, with subsequent activation of the *CCND1* promoter[222].

β-catenin activation by CagA-dependent manner has also been associated with gastric carcinogenesis. *H. pylori* CagA plays an important role in the development of intestinal metaplasia, a premalignant lesion of gastric epithelial cells from which intestinal-type gastric adenocarcinoma arises[220]. Moreover, CagA deregulates the Wnt/β-catenin pathway in a phosphorylation-independent manner by the interaction with E-cadherin and the destruction of the complex between E-cadherin and β-catenin, which causes cytoplasmic and nuclear accumulation of β-catenin. CagA-deregulated β-catenin then trans-activates *cdx1* and *p21* genes that are involved in intestinal differentiation of gastric epithelial cells[220]. Additionally, another study has suggested that non-phosphorylated CagA may induce Wnt signaling in a *met* proto-oncogene (*c-Met*)-dependent manner[223].

The relationship between *H. pylori*-induced inflammation and oncogenic mechanisms has been recently assessed by the transgenic expression of CagA in a novel

in vivo model of intestinal hyperplasia in zebrafish[224]. This study reported significant CagA-dependent up-regulation of the Wnt target genes *CCND1*, *Axin2* and the zebrafish *c-myc* ortholog *myca*. Moreover, CagA infection can lead to advanced gastric carcinoma through the upregulation of cyclin D1[225]. It is thought that CagA promotes the phosphorylation of MUC1, which is a glycoprotein expressed on the apical borders of secretory epithelial cells, thereby facilitating its interaction with β-catenin, and the subsequent transcriptional activation of cyclin D1[225]. In addition, gene expression profile analyzed in *H. pylori*-infected and uninfected patients undergoing chronic superficial gastritis showed that the E3 ubiquitin-protein ligase *RNF138*, which is involved in the ubiquitination and degradation of TCF/LEF, was downregulated by *H. pylori*[226,227].

Among the multiple ways in which *H. pylori* can induce Wnt signaling, the bacteria also induces phosphorylation of the Wnt co-receptor LRP6 in a T4SS-dependent manner and involving Dvl-2 and Dvl-3 proteins[228].

In gastric tumor mouse models, the cooperation between the canonical Wnt signaling and *H. pylori*-induced COX-2/PGE_2 pathway has been established in promoting gastric carcinogenesis *via* the metaplasia-carcinoma sequence[72,229]. In addition, the Wnt signaling pathway plays a role in the transcriptional induction of *COX2* in gastric cancer cells through a β-catenin/TCF dependent mechanism[230,231]. Furthermore, TNF-α treatment and *H. pylori* infection might play roles in human gastric cancer cell line MKN45 through upregulation of *Wnt-10A*[232].

Finally, it is important to mention the correlation between Epstein-Barr virus (EBV) and gastric cancer. In this regard, Kim *et al*[233] found that *p21* and *APC* loss was positively correlated with EPV positive gastric carcinomas. EBV is accepted as a causative microorganism for gastric carcinoma, accounting for nearly 10% of gastric cancer cases worldwide[234].

TARGETING THE WNT/β-CATENIN PATHWAY AS POTENTIAL THERAPY IN GASTRIC CANCER

The Wnt/β-catenin pathway plays a key role in gastric cancer pathogenesis; therefore, chemotherapeutic approaches for targeting this pathway should be a major effort. However, several factors have hampered progress in this field, such as the number and variety of Wnt ligands, receptors and co-receptors involved in Wnt signaling[14,18]. Furthermore, this complex pathway regulates a broad range of developmental processes, its components have redundancy in other cellular functions, and the transcriptional output of β-catenin is highly context dependent[235,236]. Finally, crosstalk from various non-Wnt factors also intervenes to regulate nuclear β-catenin accumulation.

Wnt inhibitors are the focus of intense investigation in pharmaceutical and academic research, and as a consequence of this effort; potential anticancer agents have emerged that target components of this signaling pathway[235,237-239]. There are currently several therapeutic approaches to modulate the Wnt signaling cascade at various stages of development, as well as natural compounds that have been proved, or are being tested, in different systems[235,240]. Several studies in which potential targets and strategies were tested for gastric cancer treatment targeting Wnt/β-catenin signaling pathway are reviewed below.

The antibacterial potassium ionophore Salinomycin was first described as a selective inhibitor of breast cancer[241]. Thereafter, Salinomycin was found to inhibit Wnt/β-catenin signaling by inducing the degradation of Wnt co-receptor LRP6 in chronic lymphocytic leukemia[242]. Recently, it was also confirmed that Salinomycin inhibits tumor growth by targeting preferably gastric CSCs, at least partially, *via* suppression of Wnt1 and β-catenin expression[47].

Zhang *et al*[243] examined the effects of acetyl-11-keto-beta-boswellic acid (AKBA) and its underlying molecular mechanisms on human gastric carcinoma. AKBA is an active component of the *Boswellia serrata* gum resin[243,244]. In this study, AKBA significantly inhibited human gastric cancer growth without toxicity. This effect was attributed, among other mechanisms, to induction of apoptosis and modulation of the Wnt/β-catenin signaling pathway.

Natural products involving downregulation of the Wnt/β-catenin signaling pathway have also shown antitumor effects against gastric cancer, such as γ-tocotrienol, flavanone, *Capsosiphon fulvescens* glycoprotein (Cf-GP) and diphyllin[245-248]. Diphyllin is a cytostatic lignan isolated from *Cleistanthus collinus* that potently inhibits V-ATPases involved in Wnt/β-catenin signaling[245,249]. Shen *et al*[245] observed in gastric carcinoma treated with diphyllin a decrease in phospho-LRP6, but not in LRP6, accompanied by reduction of β-catenin levels and the expression of its target genes associated with the inhibition of V-ATPase. LRP6 phosphorylation is accompanied by receptor internalization in caveolin-containing vesicles and endocytosis, which is essential for Wnt/β-catenin signaling[250].

Yao *et al*[251] observed that the combined treatment with the ERK1/2 inhibitor PD98059 and the γ-secretase inhibitor DAPT in gastric cancer originated induction of apoptosis and regulation of β-catenin, c-Myc, and cyclin D1. Although this combined treatment results in a dramatically increased cytotoxicity compared to that observed with any of the drugs alone, the exact mechanism by which these two agents act together to downregulate Wnt/β-catenin signaling pathway remains unknown.

Anti-metastatic activity resulting from disruption of Wnt/β-catenin pathway was recently reported in gastric cancer cells by Hanaki *et al*[196] using anti-Wnt-5a polyclonal antibody. They suggested that the anti-Wnt5a antibody was capable of suppressing Wnt-5a-dependent internalization of receptors, thus leading to disruption of

Wnt/β-catenin pathway. Further investigation with anti-Wnt-5a monoclonal antibody could be useful for the clinical management of cancers with overexpression of Wnt-5a. In this sense, monoclonal antibodies against Wnt-1 and Wnt-2 can induce apoptosis in some human cancers that overexpress these ligands[252,253].

Interestingly, two different approaches targeting the secreted inhibitor of the Wnt/β-catenin pathway Dkk-1 have been reported as potential treatment for gastric cancer[121,254]. As mentioned above, the role of Dkk-1 in cancer appears to be diverse, because it can act as tumor suppressor or metastasis promoter[255]. Thus, Dkk-1 appears to be critical to determining the tumor microenvironment and could has a cancer type and/or patient specific role[255]. Sato *et al*[121] reported a strategy based on Dkk-1-neutralizing antibodies indicating the direct antitumor effect in cancer cells lines that overexpressed Dkk-1, such as pancreatic, bile duct, breast, cervical and gastric cancer, and hepatocellular carcinoma. Alternatively, Wang *et al*[254] used a chimeric 5/35 adenovirus-mediated Dkk-1 overexpression strategy to suppress the endogenous Wnt/β-catenin signaling aberrantly activated in GCSC. This study demonstrated that adenoviral expression of Dkk-1 was efficient in modulating activation of Wnt signaling and also proved that CSC-targeting gene therapy could be effective in gastric cancer.

Recently, Zhang *et al*[256] reported that the proton pump inhibitor pantoprazole treatment inhibited the hyper-activated Wnt/β-catenin signaling and reduced cell invasiveness of chemotherapy-resistant gastric cancer cells and epithelial-mesenchymal transition. This work, besides giving evidence that the enhanced aggressive phenotype appeared to be mediated by activation of the canonical Wnt/β-catenin signaling pathway, it also showed that it is possible to reduce the aggressiveness of gastric cancer with a proton pump inhibitor[256].

CONCLUSION

Recent studies have identified new components and regulators, as well as confirmed other already known players, of the canonical Wnt/β-catenin pathway involved in the development, progression and metastasis of gastric cancer. These studies have also provided new approaches to therapeutically target Wnt/β-catenin signaling pathway in primary and metastatic gastric cancer. The complexity of the Wnt/β-catenin pathway and the potential influence of the context imply a critical need for understanding the interaction between Wnt regulators and with different oncogenic signaling pathways, to completely elucidate this pathway.

In this regard, the contribution of high throughput methods for functional genomics, epigenomics and for the screening of potential therapeutic molecules certainly will help in the near future. These "new generation" methods could also address the additional challenge that represents the intratumoral and intertumoral (between patients) heterogeneity for effective treatment of gastric tumors.

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REVIEW

Mood disorders: A potential link between ghrelin and leptin on human body?

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Abstract

Leptin and ghrelin are two hormones associated with multiple physiological functions, especially energy balance. Leptin is an adipocyte-secreted hormone discovered in 1950 and ghrelin which was found in 1999, is a peptide hormone produced and secreted in the stomach. A number of previous studies showed that these hormones could be associated with different types of mood disorders. The results of previous studies, nevertheless, are confounded by diverse sample selection and different methodologies. A search for related articles in the PubMed database was attempted. The search covered studies, reports, reviews and editorials published in the last ten years. Older references served as auxiliary sources for comparison purposes. However, due to the different results of the studies, there is a need for more investigation in order to establish the exact biochemical mechanisms that are responsible for these diseases and ghrelin's and leptin's effects on mood.

Key words: Mood disorders; Ghrelin; Leptin

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Core tip: Mood disorders are affecting a major amount of the world population. Considering the multiple factors that are contributing in the development of mood disorders, this review emphasizes on the role of leptin and ghrelin hormones. These two hormones have a key role in energy balance but they also have an effect on other physiological functions too, therefore we emphasize on the recent findings which raise a potential link between the hormones and the disorders of mood.

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INTRODUCTION

Mood disorders (MD) cover a wide range of mental illnesses and many studies have examined different aspects of them, such as their effect on social life of a person and how they are evolutionary changing through the years. Anxiety disorders and major

depressive disorder (MDD) are the most prevalent mental disabilities and affect a large amount of the world population[1]. The etiology of MDs is diverse. Genetic factors like family history, as well as psychological and socioeconomic features have a key role on the development of MDs[2-4]. As far as genetic factors are concerned, there has been a great deal of research on a specific gene called brain-derived neurotrophic factor (BDNF), which has an important role to the pathophysiology of MDs but which might also contribute to the therapeutic mechanisms, by exerting antidepressant effects[5,6]. BDNF is involved in neuronal plasticity, and has antidepressant effects probably due to its interaction with serotonin systems[7,8]. This interaction monitors the development and plasticity of the neural circuits which have a role in MDs. Likewise, antidepressant drugs can alter the levels of neurotrophic factors and therefore are able to contribute in neuronal plasticity[9].

Different parts of the brain are involved in the regulation of mood and the expression of emotions, as well as other functions, like reward processing[10]. Neuroimaging and neuropathological studies support that the medial prefrontal cortex, the caudolateral orbital cortex, the amygdala, the hippocampus and the ventromedial parts of the basal ganglia are networks which modulate emotional behavior[10]. There is evidence that the function of these structures is altered in patients with MDs[11-13]. For example, in cases of stress and long-term depression, the hippocampus and the prefrontal cortex are subjected to atrophy while amygdala is subjected to hyperaction[14]. Another important site of the brain that associates with MDs is the hypothalamic-pituitary-adrenal axis (HPA) which consists of the hypothalamus, the pituitary and adrenal cortex[15]. The regulation of the HPA-axis depends on the inputs that the paraventricular nucleus receives but it is also auto-regulatory. The auto-regulation is important for allostasis and it is hypothesized that allostatic change may link to MDs[15]. Moreover, a dysregulation of the HPA axis it is often connected with MDD.

The areas of the brain mentioned above are not only associated to MDs but they also have an important role in feeding behavior and nutrition hormones. Whether food choice and mood have a negative or a positive relationship depends on the psychological and the neurohormonal characteristics of a person[16]. The correlation of MDs and food intake is supported by the irregularities of the HPA axis function[17]. Danaei *et al*[18] reported that obesity has become a pandemic in the last five decades. Numerous studies have showed that obesity and MDs are related. More specifically, depression and major depression are reported as risks factors for developing obesity particularly for women[19-25].

There is evidence that obesity may be a risk factor for anxiety disorders too[26,27]. Under normal conditions, the brain is activated to confront perceived threats, by altering the HPA axis and by entering into an anabolic state. When an individual faces chronic stress, however, the metabolism shifts to catabolic state[28].

HPA axis is not the only region of the brain that is affected by MDs. The cortex, the limbic system, the midbrain and the brainstem are also subjected to changes[29]. When the physiological pathways are activated due to chronic stress, cortisol rises, and appetite hormones as well as weight are altered[30]. The reinforced motivation for food is caused by the increased secretion of glucocorticoids and which in turn leads to obesity[31].

Another hypothesis which supports the relation between obesity and MDs refers to the inefficiency of leptin signaling[32]. Leptin is a 16-kDa protein which is encoded by the obese (*ob*) gene and is mainly secreted by the white adipose tissue[33]. Leptin suppresses food intake, whereas another hormone called ghrelin stimulates appetite. Ghrelin, is a hormone secreted from stomach but it can be found in small amounts in the brain too[34]. Surprisingly, obese individuals have high circulating leptin levels, in contrast to the orexigenic hormone which is found in low levels. This suggests that obese people are leptin-resistant[35].

In this review, we focus on recent findings regarding the role of ghrelin and leptin in the regulation of MDs such MDD, and we refer to the mechanism of the pathophysiology of MD. This review points at the potential link between ghrelin and leptin on human body and MDs, as they serve as complementary hormones with multiple functions. This review takes into account information from 2004 to 2014 on the leptin and ghrelin hormones and more specifically, their function in the central nervous system (CNS), and their potential role in MDs.

LEPTIN'S ROLE IN MOOD REGULATION

Leptin has a key role in energy intake and expenditure. Specifically, circulating leptin sends information to the hypothalamus about the changes of energy intake and how much energy is stored in the adipose tissue[36]. Leptin is produced peripherally in the adipose tissue so it is essential to cross the blood-brain barrier to act inside the brain[37]. It is believed that the transportation of leptin into the brain follows a saturable system and crosses the blood-brain barrier *via* LepRs which are located in nonneural cells in the meninges, choroid and blood vessels[38,39]. The transportation of leptin becomes more intensive in the hypothalamic ARC (Arcuate Nucleus), a major site for leptin signaling and resistance[38]. Leptin affects two types of neurons in the ARC; a group of neurons secrets the protein POMC (proopiomelanocortin) together with cocaine and amphetamine-regulated transcript (CART), whereas the other class of neurons expresses neuropeptide Y (NPY) and AgRP (agouti-related protein)[40]. The NPY and AgRP peptides are expressed with the reduction of leptin, while CART and POMC are suppressed[41].

The HPA is a brain area that was systematically

Table 1 Leptin in patients with mood disorders according study's since 2004

Ref.	Findings
Westling *et al*[51]	Low CSF leptin in MDD women
Atmaca *et al*[52]	Low in OCD + D
Esel *et al*[57]	High in women but not in men
Gecici *et al*[54]	High in patients with atypical depressive disorder but not with non-atypical
Jow *et al*[50]	Low
Emul *et al*[53]	No difference
Pasco *et al*[47]	High in females non-smokers
Jimenez *et al*[46]	High in patients with post-stroke depression
Hafner *et al*[56]	High in men with social isolation and depressed
Lawson *et al*[45]	Low
Hafner *et al*[55]	High in normal-weight women
During antidepressant therapy	
Himmerich *et al*[42]	High after successful treatment

CSF: Cerebrospinal fluid; MDD: Major depressive disorder; OCD + D: Obsessive compulsive disorder + depression.

found to be associated with MDs. In depression, the overstimulation of HPA axis caused by glucocorticoid receptor resistance increase leptin synthesis and secretion[42]. Thus, studies on mouse models showed that leptin might modulate HPA function. Specifically, it seems that peripheral and hippocampal administration of leptin mitigates symptoms of depression[33,43,44]. The results of some human studies are consistent with animal studies as they showed that leptin reduces symptoms of depression and had anxiolytic effects[45]. There is a study by Jimenez *et al*[46], however, which suggests that leptin might predict post-stroke depression, as higher leptin levels were found in patients with depression after stroke[46]. Similarly, a cross-sectional study by Pasco *et al*[47], demonstrated that depressive disorders can be predicted by high serum leptin levels among female non-smokers. In addition, elevated leptin levels as well as visceral fat seem to predict depressive symptoms in old men[48]. Likewise, in a longitudinal study by Milaneschi *et al*[49] high levels of leptin and abdominal adiposity were a high risk factor for the development of depressed mood over a 9-year follow up.

Studies in humans have given controversial evidence about the role of leptin in MDs. A few studies reported that depression is associated with low leptin levels independent of body mass[45,50]. Furthermore, one study revealed that female suicide attempters with MDD had lower cerebrospinal fluid leptin than in the group with other disorders, even though both groups had similar body mass index (BMI)[51]. Patients with MDD, Obsessive Compulsive Disorder (OCD) and comorbid depression were examined in various studies. It was found that these patients had significantly lower leptin levels and higher cortisol levels than healthy controls and patients without comorbid depression and MDD[52]. Furthermore, Emul *et al*[53] found that the comorbid MDD with OCD group did not have any difference compared to patients with pure OCD and controls.

On the contrary, other studies showed that patients with different types or symptoms of depression, such as atypical depressive disorders, depressed mood and sleep disturbances as well as social isolation, had higher leptin levels than a control group[47,54-56]. Researchers who investigated the effects of leptin on antidepressant treatments found out that leptin was increased during drug treatment[42,57]. As far as anxiety is concerned, Lawson *et al*[45] found that increased symptoms of anxiety were related with low levels of leptin, independent of BMI and weight, supporting that leptin is an anti-stress hormone (Table 1).

GHRELIN'S ROLE IN MOOD REGULATION

Ghrelin is a 28-amino acid peptide, secreted from stomach and functions as an orexigenic hormone, while it is increased before meals and reduced after meals[58-61]. Ghrelin is acylated [acyl ghrelin (AG)] by ghrelin O-acyl transferase in the stomach and 10%-20% of circulating ghrelin exists in this form[59]. Acylation is required for ghrelin to activate its receptor, the growth hormone secretagogue receptor (GHSR). In particular, it promotes food intake and adiposity sending a peripheral signal to the hypothalamus through the neuropeptide Y and agouti-related protein[62]. Even though AG has a major role in energy balance it also contributes in other biological functions too, such as the regulation of insulin secretion, the regulation of glucose metabolism and influences the pituitary-gonadal axis[63].

Although ghrelin is present in the stomach and other peripheral tissues like the pancreas, it is also found in smalls amounts in the hypothalamus[34,59,63]. Therefore ghrelin affects the CNS and does not act only as a hunger signal[64]. There is evidence that plasma ghrelin passes the blood-brain barrier and binds with the hippocampus thus promoting synaptic plasticity[65]. AG activates GHSR-expressing neurons of the hypothalamus to stimulate food intake and secretion of growth hormone (GH). Ghrelin acts through the GHSR thus it stimulates the secretion of the GH from the anterior lobe of pituitary[60,66,67]. As an orexigenic hormone, ghrelin has an important role in weight gain and adiposity. Druce *et al*[61] though, found that obese people do not have different ghrelin levels that lean individuals[61]. The studies that focused on the extremes of the BMI range, found that the correlation between ghrelin levels and BMI are inversed; the lower the BMI the higher are the ghrelin levels[68]. On the contrary, leptin and ghrelin are correlated positively.

As it was mentioned already, ghrelin does not only act as a hunger signal but it is also involved in reward, motivation and signaling pathways, and is in this way linked with stress, anxiety disorders and depression[69]. Carlini *et al*[70] suggest that the relation between the

Table 2 Ghrelin in patients with mood disorders according study's since 2007

Ref.	Findings
Rouach *et al*[80]	High in patients with binge-eating disorders
Schanze *et al*[62]	No difference
Kluge *et al*[78]	No difference
Barim *et al*[68]	Low
Matsuo *et al*[79]	No difference
Ishitobi *et al*[84]	High in non-responders with MDD
Ozsoy *et al*[81]	High
Treatment and ghrelin levels	
Schmid *et al*[83]	Decrease with mirtazapine

MDD: Major depressive disorder.

limbic areas and the hypothalamus create a circuit that connects emotional states and eating behavior. Moreover, Nakashima *et al*[71], found an association between a gene polymorphism and depression. Different studies in animals yielded contrasting findings. Some showed that ghrelin could be an anxiogenic hormone whereas others revealed that it can have anxiolytic and anti-depressive responses[70,72-76]. High levels of ghrelin were observed after conditions of energy insufficiency but also after acute or chronic stress[77].

In human studies too contrasting findings were reported. Some researches did not find any differences in ghrelin levels between patients with major depression and controls[62,78,79] while other researchers suggested that plasma ghrelin levels as well as pre-proghrelin mRNA levels are elevated in conditions of stress[77,80]. Ozsoy *et al*[81], for example, found higher levels in serum ghrelin levels in depressive patients, than in controls and they also found that high serum ghrelin levels were normalized by treatment. An improvement of depressive symptoms was found in men with MDD after they were administrated with ghrelin[82]. On the other hand, a reduction of ghrelin levels in MDD patients revealed a reduction of psychopathological symptoms[83,84]. Barim *et al*[68] examined patients with depression before and after treatment with citalopram and showed that the levels of ghrelin of the depression patients were lower than the ones of healthy individuals in both periods of time. This antidepressant effect of high levels of ghrelin might be associated with the restriction of food or with hyperactivity of the HPA axis in depression and anxiety which increases the stimulation of ACTH and glucocorticoids that increase ghrelin levels[76,82-86]. The change in ghrelin levels may imply a reaction to symptoms of depression, such as loss of weight and appetite[81] (Table 2).

DISCUSSION

Leptin and ghrelin are two complementary hormones that have a major impact on energy balance. Leptin restrains gastric ghrelin to secret and inhibits the stimulation of feeding[87]. It is hypothesized that these hormones control energy homeostasis through two independent systems. Leptin functions as a feedback mechanism that signals to key regulatory centers in the brain to inhibit food intake and to regulate body weight and energy homeostasis. Ghrelin stimulates the activity of neurons expressing NPY, AgRP and orexin. Furthermore, ghrelin has an inhibitory effect on POMC neurons and CRH-producing neurons[35]. Even though leptin as well as ghrelin, exert peripheral signals, evidence from studies in animals showed that leptin is also produced in the brain and that ghrelin is synthesized in neuronal cells of the hypothalamus[88,89]. Due to their multiple functions, researchers are trying to discover their effect on MDs. Many studies showed that the mechanisms that underlie their function are critical to the development and the treatment of MDs.

Studies with patients with depression seem to suggest that that the two hormones might exert antidepressant effects, due to the change of their levels in patients with depression as opposed to controls. However, the potential antidepressant effect of the hormones is not clear, as other studies did not find any changes to serum levels between patients with depression and control and some other studies even report reverse results. Particularly, some studies revealed that high levels of the hormones are associated with depression and anxiety disorders, while others showed that depressive patients have low levels of the hormones, even before and after treatment, compared to controls. It is clear however, that the neural circuits involved in the regulation of food intake *via* ghrelin and leptin, affect emotional changes too. The systems in the CNS that are involved in energy balance are associated with the regulation of emotions[90]. The ARC of the hypothalamus and the HPA axis are two critical areas both in MDs and for the secretion and synthesis of ghrelin and leptin.

Chronic stress can lead to changes in eating patterns and metabolism, and eating and metabolism may in turn affect mood. This is only a conjecture for now as it has not been thoroughly studied to date. The researchers concluded that they had identified a previously unknown role for ghrelin in regulating mood. Results of studies have shown that ghrelin levels can be increased by chronic stress and can reduce anxiety and depression-like behaviours. These findings may be relevant to conditions such as anorexia, where ghrelin levels are known increased. Much more research will be needed to look into whether this hormone plays a role in anxiety and depression in humans.

The current review indicates that leptin and ghrelin are indeed involved in the process of MDs. The variety of methodologies used in different studies over the past 10 years might be responsible for the conflicting findings. It appears that the mechanism in which these two hormones affect mood or are affected by mood could be further enlightened if a

study is repeated controlling for dual diagnosis and comorbidity, as well as the severity of depression. Focusing on MDD can be a better indicator of biological markers since melancholia can be a result of every day events. Therefore, findings for MDD can be used as a precursor for examining other MDs with closely related symptoms.

CONCLUSION

Leptin and ghrelin do not only have a key role in energy homeostasis but they have also pleiotropic effects on the CNS and periphery. The evidence from different studies on the association between MDs and the two hormones is controversial. This can be explained by the diverse characteristics of the patients participating in the studies, the various methodologies that are used and the different measurements of the levels of hormones. As a result, the role of ghrelin and leptin in MDs is not yet clearly established. It will be interesting to examine more extensively the role of the two hormones in all of the different types of MDs in order to reveal potential common features of emotional changes and changes of the hormones levels.

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REVIEW

Asymmetric dimethylarginine, a biomarker of cardiovascular complications in diabetes mellitus

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Abstract

Cardiovascular (CV) complications are an essential causal element of prospect in diabetes mellitus (DM), with carotid atherosclerosis being a common risk factor for prospective crisis of coronary artery diseases and/or cerebral infarction in DM subjects. From another point of view, asymmetric dimethylarginine (ADMA) has been established as an inhibitor of endogenous nitric oxide synthesis and the relationship between ADMA and arteriosclerosis has been reported. In our study with 87 type 2 DM (T2DM) patients, we have examined whether ADMA and other CV risk factors are the useful predictors of DMCV complications. After the measurement of the respective CV risk factors, we have followed the enrolled T2DM patients for 5 years. We have finally analyzed 77 patients. DMCV complications developed in 15 cases newly within 5 years, and 4 cases recurred. The concentrations of ADMA in plasma were markedly more elevated in 19 DM patients with CV complications than in 58 DM patients without CV complications. Urinary albumin (U-Alb), mean intimal-medial thickness (IMT) and ankle brachial index (ABI) were also higher in patients with CV complications. Multiple regression analyses showed that U-Alb had an influence on the high level of ADMA (standardized $\beta = 6.59$, $P = 0.00014$) independently of age, systolic BP, fibrinogen, mean IMT, plaque score, and ABI. The review indicates what is presently known regarding plasma ADMA that might be a new and meaningful biomarker of CV complications in DM subjects.

Key words: Asymmetric dimethylarginine; Biomarker; Diabetes mellitus; Cardiovascular complications; Incretin

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Core tip: Asymmetric dimethylarginine (ADMA) is an emerging independent biomarker for prospective cardiovascular (CV) complications. In our study, the results show that the cases with a high level of ADMA could have diabetes mellitus CV (DMCV) complications in the future within five years. Furthermore, not only ADMA but also urinary albumin was associated with DMCV complications in the multiple regression analyses. The clinical acceptation of this parameter will rely on the availability of therapies to immediately reduce ADMA such as incretin-based drugs, which could support the part of ADMA as an etiologic risk factor.

Konya H, Miuchi M, Satani K, Matsutani S, Yano Y, Tsunoda T, Ikawa T, Matsuo T, Ochi F, Kusunoki Y, Tokuda M, Katsuno T, Hamaguchi T, Miyagawa J, Namba M. Asymmetric dimethylarginine, a biomarker of cardiovascular complications in diabetes mellitus. *World J Exp Med* 2015; 5(2): 110-119 Available from: URL: http://www.wjgnet.com/2220-315X/full/v5/i2/110.htm DOI: http://dx.doi.org/10.5493/wjem.v5.i2.110

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder and one of the common chronic diseases worldwide. The number of people with DM globally was estimated at 382 million in 2013, and is supposed to reach over 592 million by 2035[1]. Close to 5.1 million deceased in adults aged 20-79 years were attributable to DM in 2013, accounting for 8.4% of the global all-cause mortality in this age group[2]. In addition to the effect on the subjects' life quality, the microvascular [diabetic retinopathy (DR), diabetic nephropathy (DN), and neuropathy] and macrovascular complicating diseases (coronary heart diseases, peripheral artery diseases, and stroke) of DM also increase the global healthcare burden. Approximated planetary healthcare expenditure to care and preclude DM and its complicating diseases are anticipated to total leastwise 548 billion United States dollars (USD) in 2013. By 2035, this number is proposed to surpass some 627 billion USD[3]. Worldwide, DM is probable to be the fifth leading killer[4].

Cardiovascular (CV) complications are an essential causal element of prognosis in type 2 DM (T2DM), with carotid atherosclerosis (CA) being a common risk factor for prospective crisis of coronary artery disease (CAD) and/or cerebral infarction[5,6]. Some molecules, such as high-sensitivity C-reactive protein (CRP), interleukin-18, and hepatocyte growth factor (HGF) would have been presented to be atherosclerotic biomarkers[7-9]. Preclusion of DM and its involvements, early invention of disease stages, and interventions that would act in the presence of hyperglycemia to avoid, retard or inverse the involvements are the principal concerns. Biomarkers have been investigated for understanding the structures of the evolution and progress of DM involvements[10]. This review presents what is currently known regarding plasma asymmetric dimethylarginine (ADMA) level that might be a new and meaningful biomarker of DMCV complications.

ENDOTHELIAL DYSFUNCTION, NITRIC OXIDE AND ADMA

Endothelial dysfunction is distinguished as afflicted nitric oxide (NO)-mediated vascular reaction and is related to maturation of arteriosclerosis and DMCV involvements[11]. NO produced by the vascular endothelium is related to the regulatory mechanisms of the CV system[12]. Since NO is a molecule distributed to important anti-atherosclerotic properties, reduced NO availability may be deemed a crucial risk factor for atherothrombosis and acute CV events. NO is compounded by stereospecific oxidization of the terminal guanidino nitrogen of the amino acid L-arginine by the activity of a group of enzymes known as NO synthases (NOSs)[13]. The major isoform of NOS existing in endothelial cells, eNOS, is constitutively aggressive and transforms the amino acid L-arginine into NO and citrulline. Synthesis of NO can be selectively inhibited by guanidino-substituted analogues of L-arginine which blockade the action site of NOS in competition. In animals, NG-monomethyl L-arginine (L-NMMA) and NG-nitro L-arginine methyl ester have been used as pharmacological tools to reduce NO availability and induce experimental hypertension[14-16]. L-arginine analogues were identified in human plasma and urine[17]. The two L-arginine analogues identified as endogenous inhibitors of NOS were L-NMMA and NG, NG-dimethyl L-arginine (asymmetric dimethylarginine, ADMA). L-NMMA and ADMA were equipotent as NOS inhibitors[17]. Another endogenous arginine analogue (symmetric dimethylarginine, SDMA) was unable to inhibit NOS[17]. As human plasma ADMA concentrations are 10-fold higher than those of L-NMMA[17], ADMA might be regarded as the major endogenous NOS inhibitor. Increased plasma concentrations of ADMA caused impaired NO synthesis, leading to endothelial dysfunction, atherogenesis, and CV disease processes. ADMA and SDMA have been demonstrated to collect in renal failure patients[17].

Though ADMA is somewhat eliminated by the nephros, the predominant metabolism process is decomposition by the dimethylarginine dimethylaminohydrolases (DDAHs) 1 and 2 into dimethylamine and L-citrulline[18,19] (Figure 1). High blood sugar disorders DDAH efficacy in the endothelia and blood vessel involuntary muscle cells in vitro, whereby empowering raised concentrations of ADMA among DM subjects[20]. Chronic ADMA increase in animals induces arteriosclerotic involvements and nephropathy as an outcome of decreased NO production[21]. This indicates a crucial ADMA function in excusing the correlation between vascular endothelial functional impairment,

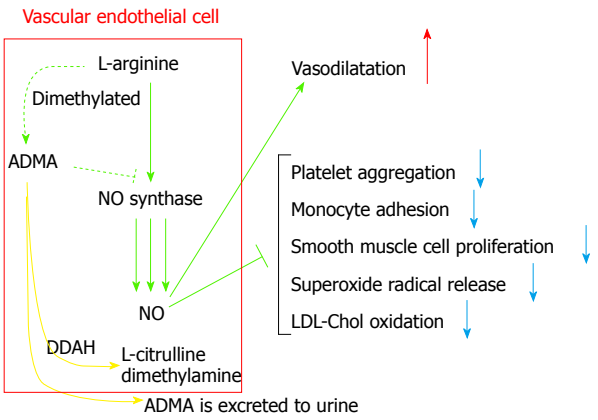

Figure 1 In non-diabetic patients, L-arginine by far outweighs asymmetric dimethylarginine, and active nitrogen oxide modulates vascular tone and structure. DDAH: Dimethylarginine dimethylaminohydrolase; ADMA: Asymmetric dimethylarginine; LDL: Low-density lipoprotein; NO: Nitrogen oxides.

atherogenesis, and DN. It has been demonstrated that ADMA is augmented in conditions such as renal impairment[17,22], DM[23], hypertension[24], and DN[25]. Moreover, it has been indicated that the concentrations of ADMA are prognosticative of CVD and all-cause death rate in preponderantly non-diabetic chronic kidney disease (CKD)[26], end-stage renal disease[27], and CAD[27-29].

PLEIOTROPIC EFFECTS OF ADMA

Vallance *et al*[17] showed that during continuous 3 mg/kg per hour ADMA infusate in *Cavia porcellus*, systolic blood pressure (BP) rose by nearly 15%, as plasma ADMA concentrations rose approximately nine-fold. In the same animal model, bolus injections of 1-30 mg/kg ADMA resulted in a dose-dependent mean arterial BP increment[17]. Gardiner *et al*[30] confirmed in rats the dose-dependent pressor effects of ADMA. De Gennaro Colonna *et al*[31] administered ADMA 10 mg/kg per day subcutaneously *via* an osmotic minipump for 14 d to male adult rats. ADMA-treated rats indicated increased systolic BP, reduced plasma nitrite/nitrate levels, NO-stable end-products and a lower vasorelaxant reaction of the aortic tissues to accumulative acetylcholine concentrations. Isolated perfused cardiac specimens from ADMA-cared rats demonstrated a deterioration of post-ischemic ventricular functional impairment[31]. In humans (healthy male volunteers), an infusion of ADMA at rates of 0.0125 and 0.025 mg/kg per minute increased plasma ADMA concentrations between 2 and 10 μmol/L and remarkably reduced plasma cyclic guanosine monophosphate (cGMP) (the main second messenger of NO) concentrations[32]. The infusion of a higher ADMA dose (0.10 mg/kg per min) importantly reduced stroke volume and heartbeat and raised systemic blood vessel resistance[32]. In another study by Achan *et al*[19], an intravenous injection of 3.0 mg/kg ADMA in healthy volunteers reduced heart rate and cardiac output and elevated BP and systemic vascular resistance. The effects of a suppressor dose of ADMA (0.25 mg/kg per min) were studied by Kielstein *et al*[33] in healthy normotensive male patients. ADMA infusion reduced plasma cGMP concentrations (indicating reduced NO production), decreased effective renal plasma flow, and elevated renovascular resistance. On the other hand, ADMA did not alter plasma renin and noradrenalin levels[33], suggesting the facility of this arginine substance to regulate renal function without impacting the renin-angiotensin and sympathetic systems. Moreover, the effects of ADMA on cerebral vascular tone in humans were researched[34]. An infusion of 0.10 mg/kg ADMA per minute in healthy males elevated vascular stiffness and reduced total cerebral perfusion[34], demonstrating that ADMA might also be related to the pathogenesis of cerebrovascular disease. In a word, respective studies demonstrate that ADMA systemic application in humans suppresses NO production and spoils endothelial action in several regions, *i.e.*, the heart, kidney, and brain.

ADMA AND CV DISEASES

Respective studies have indicated a prognosticative count of ADMA for CV outcomes. The incidence rate of CV endpoints in high risk subjects has been established to be straight and severally connected with elevated concentrations of ADMA in subjects with CAD[28], peripheral arterial occlusive disease[35], T2DM[36], type 1 DM (T1DM)[37] and chronic heart failure[38]. A specific potent relationship between ADMA and hemodynamic factors alike clinical result has been detected in pulmonary arterial hypertension subjects[39]. These forecasting information from experimental studies only reports statistic correlation and does not approve to form the consequence that ADMA is inevitable for prospective CV outcomes. It seems potential that increased ADMA levels are exclusively an epiphenomenon in parallel with other transformations. Nevertheless, animal study outcomes intimate that ADMA indicates not merely a risk biomarker but a risk factor for CV outcomes. It was demonstrated that continuous ADMA infusate for 4 wk resulted in the microangiopathy generation in murine coronary arteries[21]. Overexpression of the ADMA breakdown enzyme DDAH decreased murine ADMA and graft CAD[40]. Moreover, overexpression of DDAH avoided progress of nephropathy by blocking decrease of periductal capillary vessels and tubulointerstitium fibrosis in CKD rats[41]. Konishi *et al*[42] indicated that transgenic mice overexpressing DDAH displayed augmented endothelium rebirth and neointimas after vessel trauma. These discoveries mean that ADMA might straightly endow angiopathy. Nevertheless, it remains to be decided if a proximate ADMA level alteration can decrease CV risk in humans.

PHARMACOLOGICAL TREATMENT FOR CV DISEASES AND ADMA

A particular pharmacologic intervention to impact ADMA is not usable yet. It was demonstrated that

Table 1 Characteristic of subjects

Number of patients	87
Male/female	47/40
Age (yr)	62.6 ± 10.4
BMI (kg/m^2)	23.4 ± 4.2
HbA1C (%)	9.4 ± 2.2
Duration of diabetes (yr)	13.5 ± 10.6
With hypertension	44
With dyslipidemia	32

We enrolled 87 type 2 diabetic patients.

other steps to decrease CV hazard inclusive of survival training in T1DM subjects[43] and in increased CV hazard subjects[44] or weight reduction in pathologically corpulent subjects[45] are able to reduce the levels of circulating ADMA. It was presented that a few agents (*e.g.*, pravastatin, telmisartan or pioglitazone) are able to elevate the action or the generation of the enzyme DDAH and whereby decrease ADMA *via in vitro* experiments[46-48]. In addition, clinical studies indicated that intervention with metformin, ACE inhibitors, angiotensin receptor blockers or alpha-lipoic acid would be able to reduce the levels of circulating ADMA[49-55]. The bibliography on the efficacy of statins on ADMA is contentious[20,56-61]. Meanwhile, one clinical study demonstrated an ADMA decrease during intervention with rosiglitazone, and this was not recognized in a discrete age group[62,63]. However, it remains wondering whether regulation of ADMA by these interventions will straightly affect CV hazard additionally.

ADMA AND DM COMPLICATIONS

DM subjects have an untoward CV hazard character. Increased ADMA levels have been reported in subjects with T2DM and T1DM[23,25]. High blood sugar intrinsically may elevate ADMA levels due to decreased metabolic process. It was indicated that increased blood sugar concentrations can suppress DDAH action in cultivated vascular endothelial cells *via* an *in vitro* study[20]. In addition, clinical studies in subjects demonstrate that ADMA is straightly relevant to blood sugar concentrations[23,64]. Yasuda *et al*[65] reported that rigorous diabetic control might affect anti-atherogenicity outcomes *via* decreasing ADMA concentrations in T2DM subjects. Furthermore, there is proof that insulin resistance is related with elevated ADMA levels[50,62]. This is established by the fact that transgenic mice that overexpress DDAH have decreased ADMA levels and ameliorated insulin sensitivity measured against wild type animals[66]. Several lines of proof suggested that increased levels of ADMA were related with blood glucose control in distinct cohort which applies to the supposition that ADMA might act as a hazard predictive factor for CV outcomes[67,68]. In addition to relations between ADMA and metabolic control, the levels of ADMA might play an important part for the generation of DM involvements as well. ADMA is increased in subjects with T1DM, T2DM or DN with micro- or macroalbuminuria[25,69]. ADMA is correlated with the generation of kidney disorder and might consequently have possible harmful actions in DN subjects[26,70]. In addition, increased ADMA concentrations have been described in T2DM subjects with retinopathy[71]. Thus, it is alluring to hypothesize that ADMA might serve as a pathophysiologic suitable agent for DM involvements. Notwithstanding, high blood sugar remains a principal reason for both elevated ADMA and the generation of DM complicating disease which makes the reading of data more complicated.

ADMA AND CAROTID INTIMA-MEDIA THICKNESS

Augmented intima-media thickness (IMT) has been demonstrated to be a substitute biomarker for prognosticating CV hazard[72]. In a study by Miyazaki *et al*[73], stepwise regression analysis indicated plasma concentrations of ADMA to be remarkably related to carotid IMT. In an epidemiologic survey of 712 people, plasma concentrations of ADMA were assayed along with carotid IMT. On multiple stepwise regression analysis, carotid IMT was conspicuously related with the concentrations of ADMA[74] and the generation of carotid IMT, over a 6-year period, was associated with serum concentrations of ADMA[75]. In the PREVENCION study of 922 grown-up subjects, ADMA remarkably prognosticated carotid IMT even after adaptation for CV hazard parameters, CRP, and kidney function, but did not prefigure carotid-femoral pulse wave velocity, BP, or hemodynamic abnormality[76]. Kocak *et al*[77] determined higher ADMA concentrations in people without already-known arteriosclerotic disease who were on continuous ambulatory peritoneal dialysis and revealed a remarkable positive correlational statistics between the concentrations of ADMA and carotid IMT in these subjects.

OUR EXPERIENCE WITH ADMA

We carried out a clinical study to examine the correlation between the plasma ADMA concentrations and the stage of CA in T2DM subjects[78,79].

In our study with 87 T2DM patients (Table 1), we have examined whether ADMA and other CV risk factors are the useful predictors of DMCV complications. After the measurement of the respective CV risk factors, we have followed the enrolled T2DM patients for 5 years (Figure 2). We investigated the risk factors as follows: ADMA, angiotensin Ⅱ (AT Ⅱ), HGF, advanced glycation end products (AGEs), plasma plasminogen activator inhibitor type 1 (PAI-1), mean IMT, plaque score of the common artery and ankle brachial index (ABI). Furthermore, we measured serum creatinine (Cr), creatinine clearance (CCr), urinary albumin (U-Alb),

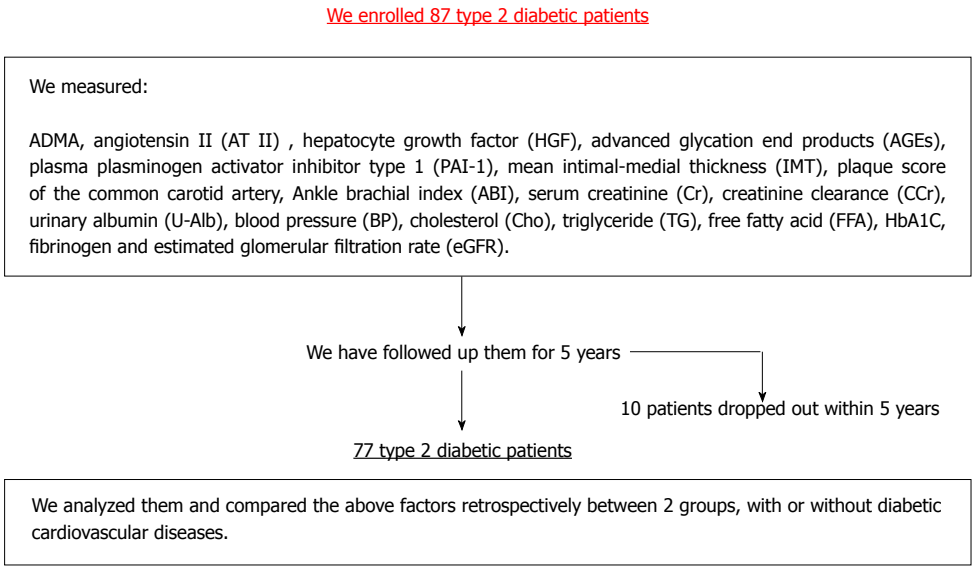

Figure 2 Protocols of our clinical research.

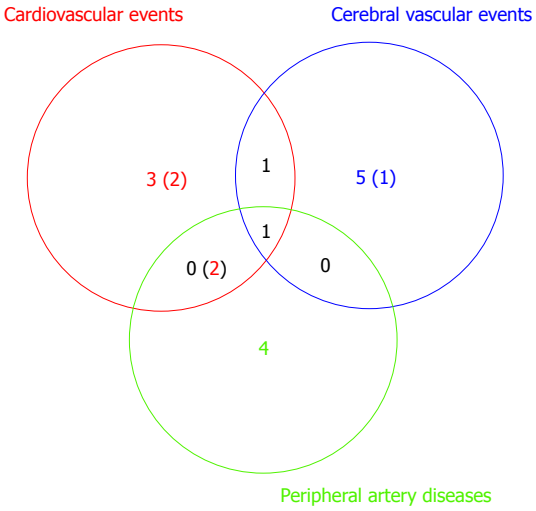

Figure 3 The result of diabetic cardiovascular disease that developed within five years. The numbers without parentheses mean new developments, and the numbers with parentheses mean recurrences.

BP, cholesterol (Cho), triglyceride (TG), free fatty acid (FFA), HbA_{1C}, fibrinogen and eGFR. We compared retrospectively the above factors between two groups with or without CV complications. In addition, we showed results in form of the average ± SD. We have finally analyzed 77 patients. DMCV complications developed in 15 cases (CV events: 5 cases; cerebral vascular events: 7 cases; peripheral artery diseases: 7 cases) newly, and 4 cases (CV events: 4 cases; cerebral vascular events: 1 case; peripheral artery diseases: none case) recurred within 5 years (Figure 3). The concentrations of ADMA in plasma were conspicuously higher in 19 DM subjects with CV complications than in 58 DM subjects without (0.56 ± 0.09 μmol/L *vs* 0.45 ± 0.07 μmol/L, $P < 0.00001$) (Figure 4). U-Alb (319.9 ± 522.6 μg/min *vs* 83.5 ± 199.4 μg/min, $P = 0.008$) and the value of mean IMT (1.39 ± 0.33 mm *vs* 1.16 ± 0.30 mm, $P = 0.006$) were also higher in patients with CV complications. The value of ABI (1.0 ± 0.2 *vs* 1.1 ± 0.2, $P = 0.046$) was lower in patients with CV complications. In the study, the other risk factors (AT Ⅱ, HGF, AGEs, PAI-1, plaque score, Cr, CCr, BP, Cho, TG, FFA, HbA_{1C}, fibrinogen and eGFR) were not associated with the development of the DMCV complications within 5 years. A relative risk of the DMCV complication development reached the highest level (6.81) when the level of ADMA was over 0.54 μmol/L. Multiple regression analyses showed that U-Alb had an influence on the high level of ADMA (standardized $\beta = 6.59$, $P = 0.00014$) independently of age, systolic BP, fibrinogen, mean IMT, plaque score, and ABI. Increased levels of ADMA were found in the generation of CA in patients with T2DM[80,81] or gestational DM[82]. Kanazawa *et al*[80] demonstrated that serum ADMA would be a predictive factor of arteriosclerosis and linked to the existence of CV complications in Japanese T2DM subjects, but serum SDMA, a structural isomer of ADMA, was correlated neither with factors for arteriosclerosis nor with the existence of CV complications.

As well as our results, Celik *et al*[83] showed that the concentration of ADMA was higher in diabetic subjects with CV complications compared to diabetic patients without complications. They also reported that the levels of fundamental determinants of ADMA were evaluated in diabetic patients with macrovascular complications, using ADMA as a dependent variable in multiple regression analysis. It was found that the most fundamental determinant of ADMA is total homocysteine (tHcy). Moreover, Krzyzanowska *et al*[84] showed that ADMA is related to clinical CV atherosclerotic disease diagnosis in T2DM, and also concluded that ADMA is associated with total tHcy, U-Alb, Cr, and GFR and that tHcy correlates with

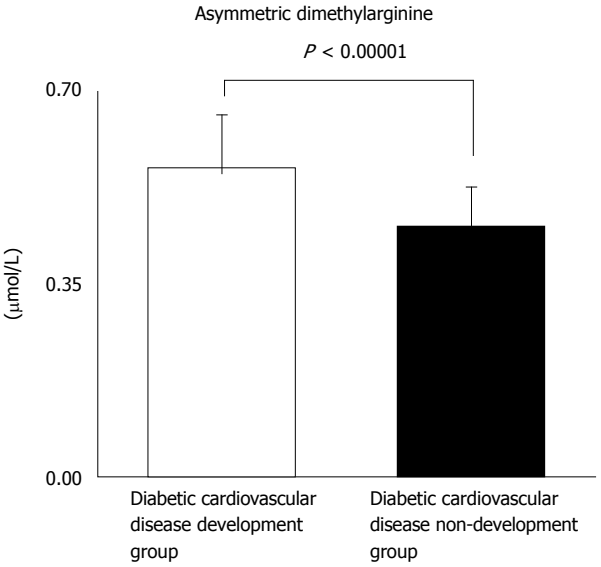

Figure 4 Asymmetric dimethylarginine was significantly higher in diabetic cardiovascular disease development group than in diabetic cardiovascular disease non-development group.

age, ADMA, Cr, GFR, and LDL[84]. Research has also shown that increased tHcy concentration in T2DM is associated with increased CV complications[85,86]. Thus, ADMA may be used to predict the likelihood of developing CV complications in DM patients.

According to these studies, the cases with a high level of ADMA, particularly those complicated with nephropathy, should be followed more carefully to prevent new developments and/or recurrences of the DMCV complications.

ADMA AND INCRETIN-BASED DRUGS

Lately, a novel treatment strategy for the intervention of T2DM that directs the incretin hormones has been generated. These peptide hormones, *i.e.,* glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide, would be secreted from the bowel after a repast and induce insulin secretion in a glucose-dependent manner[87]. Nevertheless, their activity is restricted by prompt deactivation *via* the enzyme dipeptidyl peptidase (DPP)-4. Furthermore, T2DM subjects normally do not react well to glucose-dependent insulinotropic peptide and GLP-1[88,89]. Suppression of DPP-4 will elevate levels of active incretins, so DPP-4 has turned out to be a marker in diabetic control[90-92]. Incretin-based therapy was first made available for the treatment of T2DM in the United States in 2006 and in Japan in 2009[93]. Up to now, seven DPP-4 inhibitors are usable in Japan, including sitagliptin, vildagliptin, alogliptin, linagliptin, anagliptin, teneligliptin, and saxagliptin[93-95]. The elevated intrinsic plasma level of GLP-1 is considered to show protective outcomes on the CV system[96]. Ojima *et al*[97] showed that GLP-1 receptor agonist would inhibit ADMA development in the kidney of streptozotocin-induced DM rats. In addition, serum concentration of ADMA was sought with the DPP-4 inhibitor saxagliptin in an animal experiment[98]. The discoveries of that study indicate that the DPP-4 inhibitors could impact serum concentrations of ADMA. In fact, several DPP-4 inhibitors might reduce ADMA levels in T2DM subjects[99,100].

Incretin-based drugs were found to decrease serum concentrations of ADMA in T2DM subjects. This discovery ought to be backed up with larger-scale prospective randomized trials such as Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus-Thrombolysis in Myocardial Infarction 53 study[101,102] and EXamination of cArdiovascular outcoMes: AlogliptIN *vs* standard of carE in patients with type 2 diabetes mellitus and acute coronary syndrome trial[103] to conclude that incretin-based drugs provide CV protection along with DM regulation.

CONCLUSION

ADMA is a rising distinct biomarker for prospective CV accidents. The clinical adoption of this factor will rely on the accessibility of therapies to straightly lower ADMA such as incretin-based drugs, which could support the function of ADMA as a prolific risk factor. Additional studies would be guaranteed in DM patients especially concerning the possible effects of ADMA on DMCV complicating diseases.

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MINIREVIEWS

Electrophysiological neuromonitoring of the laryngeal nerves in thyroid and parathyroid surgery: A review

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Abstract

Recurrent laryngeal nerve (RLN) injury is one of the most common complications of thyroid surgery. Injury to the external branch of the superior laryngeal nerve is less obvious and affects the voice variably; however, it can be of great significance to professional voice users. Recent literature has led to an increase in the use of neuromonitoring as an adjunct to visual nerve identification during thyroid surgery. In our review of the literature, we discuss the application, efficacy and safety of neuromonitoring in thyroid surgery. Although intraoperative neuromonitoring (IONM) contributes to the prevention of laryngeal nerves injury, there was no significant difference in the incidence of RLN injury in thyroid surgery when IONM was used compared with visual identification alone. IONM use is recommended in high risk patients; however, there are no clear identification criteria for what constitutes "high risk". There is no clear evidence that IONM decreases the risk of laryngeal nerve injury in thyroid surgery. However, continuous IONM provides a promising tool that can prevent imminent nerve traction injury by detecting decreased amplitude combined with increased latency.

Key words: Neuromonitoring; Superior laryngeal nerve; Recurrent laryngeal nerve; Thyroid surgery

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Core tip: As recurrent laryngeal nerve injury is one of the most common causes of medicolegal litigation after thyroid and parathyroid surgery, securing the nerve is an increasing demand in these surgeries. Although visual identification has been used as the gold standard and had been proved to reduce the rate of laryngeal nerves injury, intertwining with inferior thyroid vessels and unusual course of the nerve may be challenging. Neromonitoring has been introduced as a novel technique to help identifying the nerve and prevent misidentification of any cord-like structure as a nerve, the thing that can reduce rate of laryngeal nerves injury.

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INTRODUCTION

Visual identification of the recurrent laryngeal nerve (RLN) is considered the gold standard in the prevention of nerve injury during thyroid surgery[1-3]. Although visual identification of RLN during thyroid surgeries decreases the rate of permanent RLN injury, it remains the most common cause of medicolegal litigations after these surgeries[4].

In addition to the morbidity caused by bilateral vocal cord paresis, monetary settlements to plaintiffs can reach up to millions of dollars[5]. The RLN is at higher risk of injury during thyroidectomy in high risk patients, including patients with substernal goiter, advanced thyroid cancer, or patients undergoing reoperation[6,7].

Intra-operative nerve monitoring (IONM) technologies were first reported to decrease the risk of RLN injury by Shedd and Durham in 1965[8]. Intramuscular vocal cord electrodes were described by Basmajian in 1970[4]. It was proposed that IONM could be useful in nerve identification and prevention of RLN injury in thyroid surgery[2,6,9]. However, IONM was not proven to decrease the rate of permanent RLN palsy[10,11]. Since IONM has been introduced, it has gained popularity, especially in the last decade[12]. It was reported that over recent years, about 65% of otolaryngologists and 53% of general surgeons use IONM in some or all of their cases[13,14].

Additionally, IONM has been reported to be a safe and feasible adjunct to the routine visual identification of the laryngeal nerves[7,15]. Some studies showed that IONM decreases the prevalence of transient RLN injury[6,16,17]. This may be due to its ability to aid in the dissection of the RLN near the Berry's ligament, as it enhances the detection of branched nerves and reduces traction injury of the anterior branches of these nerves[16]. Another study reported that IONM is effective in video-assisted thyroidectomy as it makes surgeons more comfortable[18].

In contrast, studies comparing IONM to the routine visual identification of RLN showed no statistically significant differences in the rate of overall, transient or permanent RLN palsy[10,19-22], but this may simply be due to the low incidence of RLN palsy. It has been reported that at least 39907 nerves at risk for both groups, either IONM or visual identification, are needed to show a significant difference in the nerve injury rate[2].

IONM did not show much improvement with experienced thyroid surgeons, with an RLN injury rate of < 1%[6]. However, it showed a decrease in the incidence of permanent RLN palsy in the hands of low-volume surgeons[2]. Studies have also recommended the use of IONM in patients whose laryngeal nerves are at higher risk of injury[7,21]. A single study analyzed the performance of IONM in repeat thyroid surgeries and found that it did not reduce the incidence of RLN injury in these patients[23].

Sari *et al*[24] showed that IONM decreases operative time when compared to visualization alone by shortening the time needed to identify the RLN. However, a systematic review reported no difference in operative time when IONM was used compared to visual identification alone[22].

It was reported that IONM can prognosticate the postoperative status of the RLN as an intact non-reduced monitoring signal at the end of the operation. This is consistent with a negative predictive value higher than 97%[2,6]. However, with loss of signal, the positive predictive value was reported to be as low as 33%-37.8%[2,6]. It is recommended that contralateral surgery be avoided if loss of signal is encountered in the first lobe resection[6].

Though IONM is considered a safe procedure, it carries some limitations. It is difficult to determine whether the change in EMG amplitude is due to nerve injury or loss of contact between vocal cords and recording electrodes. In addition, EMG signal can also be affected by anesthesia and manipulation of trachea.

Intermittent IONM assessment of nerve integrity is limited to the short interval of direct stimulation. Hence, RLN palsy is usually detected after it has occurred. It is also limited to testing the part of the nerve distal to the point of nerve stimulation, thus it can easily miss proximal nerve injury, or injury in the gap between 2 stimulations.

Continuous IONM was introduced as a superior modality that can avoid periodic short timed stimulation and more accurately detect proximal injuries. A clip is placed over the vagus nerve and stimulates it periodically at short intervals. This allows it to recognize any lesion along the RLN or external branch of the superior laryngeal nerve (EBSLN), even the most proximal[25]. By providing vital intraoperative information about impending RLN injury caused by suture compressing the nerve or traction, continuous IONM allows surgeons to take measures to reverse the adverse condition and save the nerve. Traditional stimulator probes should still be used in conjunction with the continuous IONM system as it helps in mapping the nerves.

Supporters of IONM claim that its use helps to protect the RLN and EBSLN by detecting them before visualization, providing information about nerve function during surgery, and by detecting anatomical variants that are at higher risk of injury[3]. However, some disadvantages include possible technical failures such as electrode displacement, and a high rate of false positive results leading to unnecessary staging in bilateral surgeries[3].

CONCLUSION

IONM has some advantages over visual identification

alone. Most injured nerves appear intact by visual inspection, but IONM provides a more accurate prediction of postoperative neural function. In addition, IONM helps avoid bilateral RLN injury by staging the operation if a nerve injury is encountered in the initial side of a bilateral operation. Continuous IONM is a promising improvement over traditional monitoring as it can give timely information about nerve status which may reverse impending nerve injury. Additionally, it can detect proximal nerve injury which might be missed by intermittent IONM of the RLN. Although some studies showed that IONM decreases the rate of RLN palsy, most of the studies reported no statistically significant difference between it and visual identification regarding laryngeal nerves injury. Due to the low incidence of permanent RLN palsy, more controlled studies with larger populations are warranted to provide a better assessment of this technique in preventing laryngeal nerve injury.

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Genetic test in multiple endocrine neoplasia type 1 syndrome: An evolving story

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Abstract

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant inherited tumour syndrome expressing various endocrine and non-endocrine lesions and tumours. Since the identification of the causative gene, the oncosuppressor gene *MEN1*, in 1997, genetic testing has revealed an important approach for the early and differential diagnosis of the disease. The finding of a *MEN1* mutation in a patient has important clinical implications for relatives since it allows very early disease diagnosis and identification of carriers, even before biochemical and/or clinical manifestation, permitting their inclusion in a specific program of surveillance and subsequent praecox therapy. Currently, genetic testing for MEN1 consists principally of the sequencing of coding regions and intron-exon junctions of the *MEN1* gene. However, the recent acquisition of novel high throughput technologies will allow the design of innovative, accurate, complete and rapid genetic diagnosis. These new tools are able to increase the strength of the analysis and almost completely eliminate the possibility of false negative results. This review aims to give an overview on genetic testing of MEN1 syndrome, reporting the positive aspects of performing the analysis and the future perspectives for improving the performance of the test, as well as its application in clinical practice.

Key words: Multiple endocrine neoplasia type 1; Genetic test; Clinical practice; Next-generation sequencing

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Core tip: Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant inherited tumour syndrome. Mutation analysis of the causative gene, the oncosuppressor *MEN1* gene, is today the best approach for the early and differential diagnosis of the disease. The manuscript gives an overview on current genetic testing of MEN1 syndrome, reporting the positive aspects and clinical utility of performing the analysis. Moreover, the paper aims to report the future perspectives for improving the performance of the test and its application in clinical practice.

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INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100) is a rare (approximately one in 30000) autosomal dominant inherited tumour syndrome with high penetrance (nearly 100% by the age of 50), principally affecting parathyroid glands, entero-pancreatic neuroendocrine tissues and anterior pituitary[1]. A variable combination of about 20 different endocrine and non-endocrine lesions and tumours can randomly occur in families and sporadic cases of the syndrome; despite the heritability of the disease, clinical features may differ in members of the same family and even between homozygote twins[2]. A MEN1 case is clinically defined in a single patient showing at least two of the three main tumours or in a familial case presenting with one of the main syndrome-related tumour and one first-degree relative affected by the syndrome.

MEN1 syndrome occurs as the result of tumour growth and associated metastases which cause the overproduction of hormones. MEN1 patients have been shown to have a reduced life expectancy; approximately 30% to 40% of deaths are associated with malignant neuroendocrine pancreatic tumours (mainly gastrinomas) and thymic or bronchial carcinoid tumour (patients affected by these malignancies have a three-fold higher risk of death)[3]. The improved management of MEN1-associated primary hyperparathyroidism and hypergastrinemia, as well as prophylactic ablation of thymus, have contributed to reduce the mortality of the syndrome. Paradoxically, the longer life expectancy could result in an increased risk for MEN1-associated malignancies. Indeed, current treatments, which are usually the same used for the sporadic tumour counterparts in non-MEN1 patients, are often not as successful in MEN1 patients because of the presence of multiple tumours in different organs, tumours that are generally more aggressive, larger and presenting with recurrences and metastases. An early diagnosis of the disease associated to pre-symptomatic tumour detection and specific therapy, starting as soon as possible, are, at the moment, the best and most effective way to grant MEN1 patients a better prognosis and a reduction of morbidity and mortality. The medical diagnosis of MEN 1 is commonly made by reconstruction of family history, physical examination of signs and symptoms of excess hormones, as well as specific biochemical screenings and imaging tests. These clinical and instrumental approaches usually allow a MEN1 diagnosis only during the fourth or fifth decades of life of the patient, delaying the performance of targeted surgical procedures and/or the initiation of available therapies. Biochemical detection of serum and/or urinary variations in hormones or other molecules deregulated by the presence of one or more MEN1-associated tumour can anticipate the disease diagnosis and tumour detection by about 5-8 years with respect to clinical onset and manifestation[4]. The early recognition of affected subjects and of at-risk individuals within a family can be further anticipated by DNA-testing. Indeed, since the identification of the causative gene, the oncosuppressor gene *MEN1* at the 11q13 region, the genetic test, through *MEN1* gene mutational analysis, has been revealed to be a fundamental approach for early and differential diagnosis of MEN1 syndrome. The advantages of genetic testing are: it requires only a single blood sample, it can be performed at any age and, in theory, it does not need to be repeated during the patient's lifetime. This paper provides a general overview on the current genetic approaches to MEN1 diagnosis, reporting the positive aspects and clinical utility of performing this analysis and explaining the future perspectives for improving the performance of the test and its application in clinical practice.

GENETIC TEST IN MEN1: GENETIC BASIS FOR CLINICAL MANAGEMENT OF PATIENTS

MEN1 syndrome is caused by inactivating mutations of the *MEN1* gene, encoding for the nuclear protein menin. MEN1 exhibits an autosomal dominant pattern of inheritance, and each affected patient has a 50% probability of transmitting the genetic defect and disease predisposition to offspring, independent of sex. Also, all the first-degree relatives of a mutation carrier have a 50% risk of inheriting the mutation and developing the disease. The proposed model for tumorigenesis in MEN1 was according to Knudson's "two hit" hypothesis for tumour suppressor genes[5]. A mutated allele of the *MEN1* gene is inherited from the affected parent or developed at embryonic level (first hit); the second wild type allele is lost at somatic cell level (second hit). Tumours develop after inactivation of both *MEN1* copies at chromosome 11q13, with acquisition of a homozygous recessive state at the tissue level. The absence of wild type menin is responsible for tumour development, but the exact molecular mechanisms of MEN1 tumorigenesis must still be completely clarified and are strongly suspected to be tissue-specific[6]. Moreover, the great clinical variability even between members of the same MEN1 family and/or between patients bearing the same *MEN1* mutation may lead to suspicion regarding the influence of other genetic (*i.e.*, the contemporaneous presence of genetic variations and polymorphisms in genes other than *MEN1*) and non genetic tumour-predisposing cofactors[7]. The presence of a *MEN1* mutation is associated with the development of the

syndrome with a nearly complete penetrance by the age of 50[8]. To date, no cases of mutation carriers who have not developed at least one of the MEN1-associated tumours during their lifetime have been described. Recently, a novel mutation in exon 2 of the *MEN1* gene (Leu143His) has been associated with a limited penetrance and a specific phenotype; family members bearing the mutation presented a mild or even absent primary hyperparathyroidism and multiple well-differentiated functionally active neuroendocrine tumours of the pancreas[9].

In general, the identification of a *MEN1* mutation represents a positive genetic diagnosis of MEN1. Mutation analysis of the *MEN1* gene allows an early identification of subjects who will develop MEN1 syndrome and it is strongly recommended for: (1) index cases meeting the clinical criteria for MEN1 syndrome (sporadic case affected by at least two of MEN1-related main tumours or individual with one of the MEN1-related main tumour and with a first-degree relative affected by at least one of MEN1-related main tumours); (2) index cases not fully meeting clinical criteria for MEN1 but with two or more MEN1-related lesions and/or recurring tumours; and (3) asymptomatic first-degree relatives of a patient with an identified *MEN1* mutation.

Before the discovery and positional cloning of the *MEN1* gene in 1997, the genetic ascertainment of the syndrome consisted of haplotype analysis, using microsatellite markers at the 11q13 locus, in MEN1 affected kindred[10]. The analysis consists of a polymerase chain reaction (PCR)-based haplotype analysis of a series of microsatellite markers flanking, both at centromeric and telomeric sides, the 11q12-13 region containing the *MEN1* gene. Some of these markers exhibit recombination 0 with the *MEN1* gene (*i.e.*, PYGM, D11S463, D11S427) granting the test for carriers to reach up to 99.5% of accuracy and excluding incorrect results due to meiotic crossing over. The analysis requires the availability of a MEN1 family with at least two living clinically affected members, spanning at least two generations, and it allows for the identification, in affected individuals, of the family-specific 11q13 risk haplotype. This screening presents the limitation that it cannot be applied to a single index case. With the reconstruction of haplotypes of all the members of the family it is possible to assess who inherited the predisposing 11q13 haplotype. Since the discovery of the *MEN1* gene this kind of diagnostic approach has been replaced by the PCR-based sequencing analysis of the gene itself. However, the haplotype analysis is still useful and is suggested for the identification of gene carriers in MEN1 families in which a *MEN1* gene mutation has not been identified.

After the positional cloning of the *MEN1* gene and its identification as the gene for MEN1 syndrome, the genetic diagnosis of MEN1 consists in the mutation analysis of the coding regions (exons 2-10) and intron-exon junctions of this gene. The great majority of laboratories worldwide currently use selective PCR-based amplifications of *MEN1* exons and splicing sites, followed by specific PCR-based incorporation of dideoxynucleotides according to Sanger sequencing strategies. The *MEN1* gene consists of 10 exons encoding for a main mRNA transcript of 2.8 Kb. Less commonly mRNA transcripts have been also described, presenting variations of the 5'-untranslated region but not of the coding region[11]. The entire exon 1 (in some cases referred as two distinct exons, as reported on the NCBI Gene database: Gene ID: 4221) and part of exons 2 and 10 are non coding regions, and, thus, exon 1 is conventionally non-included in the mutation screening. Mutations in the *MEN1* promoter region have not been described[5]. About 70%-90% of MEN1 families and sporadic cases result to be carriers of a *MEN1* mutation; some studies reported that 5%-10% of patients do not have an identifiable mutation in the coding region or in splice sites of the gene[7]. They may have mutations in the promoter, introns or untranslated regions that normally are not analysed in genetic tests, or they may bear a gross gene deletion/insertion that cannot be identified by the classical *MEN1* sequencing analysis. Large gene deletions, insertions or other gross rearrangements can be detected by Southern blot analysis or by other gene dosage procedures. Multiplex ligation-dependent probe amplification (MLPA) is a quantitative highly sensitive and accurate multiplex PCR technique able to detect copy number changes within a gene and, thus, identify the presence of entire exons and/or whole gene loss or other gross intra-genic modifications. MLPA diagnostic screening should be considered in MEN1 index cases with a negative *MEN1* sequencing test, to detect large *MEN1* coding region deletions/duplications. In case of both sequencing and MPLA negative screenings, the application of familial haplotype analysis should be considered[12].

Patients with MEN1-like clinical phenotypes but negative to *MEN1* genetic tests may also represent phenocopies. Phenocopies may mimic MEN1 either by occurrence of a single sporadic endocrine tumour within a MEN1 kindred or of two endocrine tumours/lesions associated with different aetiologies and, thus, they can confound clinical diagnosis of the disease. It is estimated that phenocopies may arise in up to 5% of MEN1 families[13]; their recognition, also by specific genetic tests, is very important in the clinical management of MEN1 patients. In particular, the multiple neoplasia type 4 (MEN4) syndrome, a rare hereditary tumour syndrome presenting clinical manifestations overlapping with those of MEN1 but caused by inactivating mutations of the *CDKN1B* gene, should be strongly suspected, and the *CDKN1B* gene should be screened for mutations. Indeed, to date, nine different inactivating mutations of the *CDNK1B* gene (including missense, nonsense and frameshift variations and mutations in the 5'UTR of the gene) have been identified in 9 patients with a clinical MEN1-like syndrome but without any identified

MEN1 mutation[14]. However, because of the rarity of the MEN4 syndrome, the very small number of *CDKN1B* mutated patients, and the still unclear data about molecular mechanisms underlying MEN4 tumorigenesis, specific surveillance programs and specific clinical and therapeutic guidelines are not yet available for this syndrome, and the clinical management of patients overlaps that of MEN1 syndrome. Recently Longuini *et al*[15] found an association between the *CDKN1B* rs2066827 polymorphic variant and tumour multiplicity in patients bearing a *MEN1* germline mutation. In MEN1 patients over 30 years of age with a *MEN1* truncating mutation, the presence of rs2066827 T allele was strongly higher in subjects with susceptibility to tumours in multiple glands (3 or 4 affected glands *vs* 1 or 2 affected glands). These data need to be confirmed by additional studies and by functional analyses but they seem to suggest that the *CDKN1B* gene may act as a disease modifier in MEN1 syndrome in association to *MEN1* mutation, influencing the severity of disease clinical outcomes. In addition, less than 2% of clinical MEN1 patients without *MEN1* mutation may have mutations or polymorphisms in members of the cyclin-dependent kinase inhibitor (CDKN) family, other than *CDKN1B*, such as *CDKN1A, CDKN2B*, or *CDKN2C* genes encoding, respectively, the $p21^{cip1}$, $p15^{Ink4b}$, $p15^{Ink4c}$ proteins, which all negatively regulate the cell cycle progression and cell growth[7]. Genetic analyses of these genes should be considered in patients with classical clinical manifestations of MEN1 but have resulted negative for *MEN1* and *CDKN1B* mutations.

Lemos and Thakker[7] in 2008 created a database of published *MEN1* mutations by searching the NCBI PubMed scientific publication database for article in English. No uploaded mutation database has been published or released after that. They reported a total of 1133 different germline and 203 somatic mutations. Twenty-four polymorphisms (12 in the coding region, of which 10 synonymous and 2 non-synonymous, 9 in the introns and 3 in the untranslated exonic regions) have also been described. *MEN1* mutations are scattered along the entire 1830 bp coding region without showing any hot spot, making genetic analysis labour-intensive. Mutations are different in their types: 20% missense mutations, 23% nonsense mutations, 41% frameshift insertions or deletions, 6% in-frame insertions or deletions, 9% splice site mutations and 1% large deletions. About 80% of all identified *MEN1* mutations are responsible for a truncated protein unable to reach the nucleus of the cells and exerting its role of oncosuppressor[1].

Unfortunately, no genotype-phenotype correlation has been reported, and the identification of a specific *MEN1* mutation and/or a specific mutated region of menin does not allow the prediction of clinical outcomes of the disease, strongly reducing the importance of genetic information for the individual clinical management of both affected and asymptomatic mutation carriers. However, the individuation of any *MEN1* mutation in an individual gives indications for a lifelong routine of clinical surveillance for MEN1-associated tumours and lesions, indicates some surgery procedures and enables the specific mutation analysis for first-degree relatives in order to individuate mutation carriers. In the presence of a *MEN1* mutation lifelong specific clinical surveillance is suggested, as specifically reported in Table 1. These guidelines are reported according to the most recent "clinical practice guidelines for Multiple Endocrine Neoplasia type 1" drafted by the leading worldwide specialists in this field[16]. Moreover, a positive test can also determine the type of surgical intervention for MEN1-associated primary hyperparathyroidism, leading to the decision for a total parathyroidectomy to prevent future recurrences of parathyroid adenomas, instead of the selective ablation of only the adenomatous gland/glands in subjects with a negative *MEN1* genetic test. In *MEN1* mutation carriers also a prophylactic thymectomy is also strongly suggested, at the same time as the parathyroid surgery, to prevent the occurrence of thymic carcinoids and related malignancies and to remove possible intra-thymic ectopic/supra-numerary parathyroid glands. A positive test may also direct prenatal decisions, such as the performance of *in vitro* fertilization with pre-implantation genetic selection of non-mutated embryos or *MEN1* prenatal genetic test in a naturally occurring foetus. Conversely, a negative test in a MEN1 family member is an indication for no further biochemical and instrumental MEN1-related screenings and for no prenatal genetic testing.

FUTURE PERSPECTIVES IN *MEN1* GENETIC TEST

As mentioned above, the great majority of laboratories worldwide performing the genetic test for MEN1 syndrome, uses PCR-amplification of exons and splicing sites, followed by the PCR-based Sanger sequencing method. Only some of these laboratories also perform MPLA or linkage analyses in case of a negative sequencing result. These approaches fail to detect possible mutations in non coding and regulatory regions as well as identify phenocopies. The identification of possible phenocopies for the correct differential analysis of tumour endocrine syndromes would require a sequential traditional Sanger sequencing of all the associated genes, an extremely time-consuming approach. Next generation sequencing (NGS) is a novel sequencing technology that could allow these limitations to be bypassed, increasing the strength, efficacy, completeness and speed of genetic analysis. Application of NGS to the genetic diagnosis of the disease permits, at the same time, the sequencing of the coding regions, the introns, the untranslated and the regulatory regions, allowing the large intra-genic deletions/duplications to be recognised and the identification of novel

Table 1 Suggested guidelines for biochemical and instrumental surveillance screenings in individuals bearing any *MEN1* gene mutation

Tumour/lesion	Recommended age of begin screenings (yr)	Biochemical screenings	Frequency	Instrumental screenings	Frequency
Parathyroid adenoma	8	Fasting total serum calcium concentration (corrected for albumin) and/or ionized-serum calcium concentration Fasting serum concentration of full-length PTH	Yearly	None	NA
Gastrinoma	20	Fasting serum gastrin concentration	Yearly	None	NA
Insulinoma	5	Fasting serum glucose and insulin concentration	Yearly	None	NA
Other pancreatic neuroendocrine tumours	Under 10	Serum concentration of chromogranin-A, glucagone, pancreatic polypeptide, vasoactive intestinal peptide	Yearly	Abdominal CT, MRI or EUS	Yearly
Anterior pituitary adenoma	5	Serum concentration of prolactin and insulin-like growth factor-1	Yearly	Head MRI	Every 3-5 yr (depending on results of biochemical screenings)
Adrenal gland tumour	Under 10	None unless symptoms or signs of functioning tumour and/or tumour > 1 cm are recognised by imaging	NA	Abdominal CT or MRI	Yearly with pancreatic imaging
Thymic and bronchial carcinoid	15	None	NA	Thoracic CT or MRI	Every 1-2 yr

CT: Computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasound; NA: Not applicable.

unexpected causative mutations. This method allows the enlargement of nucleotide sequencing from single-gene to multi-gene-disease-targeted panels and up to the entire genome using platforms and instruments capable of producing hundreds of gigabytes of genetic data in a single run. The technique gives a less-expensive and higher-throughput alternative to DNA sequencing compared to the traditional Sanger method. Moreover, NGS provides a high degree of flexibility for the level of resolution required for any single genetic analysis and a sequence run can be specifically tailored to produce more or less data and/or to screen one or more preselected regions of the genome or a specifically designed set of genes. Two alternative approaches for the detection of gene mutations are available: (1) the sequencing of the entire human genome, which facilitates the discovery of genes and regulatory elements, even unknown, associated with diseases; and (2) NGS targeted multi-gene sequencing, using a platform including a selected panel of genes (usually including all the coding, non coding and regulatory regions of each gene), which allows the identification of disease-causing mutations for the genetic diagnosis of a specific disease or a group of related disorders (*i.e.*, hereditary endocrine tumours). The first approach is suggested for diseases with a suspected genetic origin but for which the causative gene/genes are not yet known. The second approach will be useful for the differential genetic diagnosis of hereditary endocrine tumours by the design of a specific platform including all the genes associated, up to date, to these pathologies.

In 2013 Rattenberry *et al*[16] tested a targeted NGS platform, including 9 causative genes, for the genetic diagnosis of patients with pheochromocytoma and paraganglioma, allowing this NGS-based test to be established, validated and introduced into diagnostic practice. Very recently Welander *et al*[17] enlarged the targeted NGS platform for the mutation analysis of pheochromocytoma and paraganglioma, including 14 different susceptibility genes, proving to be a cost- and time-reducing effective genetic diagnostic method for these tumours. A similar NGS-targeted approach was applied to the detection of mutations in thyroid cancer (ThyroSeq panel including 12 cancer genes with 284 mutational hot spots)[18], and also in this case NGS was revealed to be an optimal genetic test for multiple genes with high accuracy and very good sensitivity.

These promising results suggest possible development and application of an NGS targeted platform for the mutation analysis of multiple endocrine neoplasia syndromes and inherited endocrine tumours in the near future. The classical Sanger monogenic sequencing method should be maintained as a pre-screening test in all the suspected MEN1 probands and also for the research of a specific mutation in relatives of *MEN1* mutated individuals, while targeted NGS analysis should be applied routinely to patients showing clinical manifestations of MEN1 but resulting to be negative from classical Sanger sequencing of the *MEN1* and the *CDKN1B* genes.

CONCLUSION

The possibility of genetic testing has facilitated increased praecox and specific diagnosis of MEN1 syndrome. Even if it is a rare disease, and despite the fact that the presence of a specific *MEN1* mutation does not allow the prediction of clinical phenotype, the

finding of a *MEN1* mutation in a patient has important implications for his/her clinical surveillance and for clinical management of first-degree relatives. The application of genetic diagnosis, and the subsequent early identification of at-risk individuals have contributed, in the last two decades, to the reduction of morbidity and mortality of patients.

A genetic positive test, predicting the future development of MEN1, may ultimately have an important psychological impact on the proband and his/her family, often causing distress, anxiety, depression, *etc*. Clinical practice guidelines for MEN1[16] recommend that MEN1 patients and their families be followed by a multidisciplinary team of specialists, with experience in the diagnosis and treatment of endocrine tumours, including genetic counselling before and after the genetic tests are carried out. The genetic counselling, performed by a professional from the discipline of medicine or genetics, should include the correct and detailed explanation of psychosocial benefits and risks of the genetic test result, as well as its influence in clinical management and quality of life of the patient and his/her relatives. In general, collaboration between genetic counsellors, laboratories and clinicians could help a patient (and his/her family) to understand the clinical meaning of his/her genetic test result, whether positive, negative, or indeterminate, and better plan preventive screenings and therapeutic approaches.

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MINIREVIEWS

Does physical therapy and rehabilitation improve outcomes for diabetic foot ulcers?

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Abstract

One of the most common and serious complications of diabetes mellitus is ulceration of the foot. Among persons with diabetes, 12%-25% will present to a healthcare institution for a foot disorder during their lifespan. Despite currently available medical and surgical treatments, these are still the most common diabetes-related cause of hospitalization and of lower extremity amputations. Thus, many adjunctive and complementary treatments have been developed in an attempt to improve outcomes. We herein review the available literature on the effectiveness of several treatments, including superficial and deep heaters, electro-therapy procedures, prophylactic methods, exercise and shoe modifications, on diabetic foot wounds. Overall, although physical therapy modalities seem to be useful in the treatment of diabetic foot wounds, further randomized clinical studies are required.

Key words: Diabetic foot; Ulcer; Treatment; Physical therapy; Rehabilitation

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Core tip: People with diabetes are prone to frequent and often have severe foot problems. Treatments for diabetic foot ulcer (DFU) include surgical debridement and drainage, antimicrobial therapy for infected wounds, pressure off-loading methods and advanced wound dressings. Thus, many clinicians and researchers have made efforts to develop adjunctive or complementary treatments to improve the outcome of DFUs. This paper presents a review of the epidemiology, pathogenesis and clinical manifestations of DFUs, and a discussion of the data available on relevant physical therapies and rehabilitation methods.

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INTRODUCTION

Diabetes mellitus is a growing problem worldwide and now affects about 5%-15% of the entire population

in many developed and developing countries[1]. While diabetes can lead to complications in many organ systems and ulceration of the foot is now among the most common and serious complications[2]. An estimated 12%-25% of persons with diabetes will present to a healthcare institution for some type of foot disorder during their lifetime[3-5]. Most often these are ulcers that arise largely as a consequence of peripheral neuropathy and, to a lesser degree, peripheral arterial disease. Diabetic foot ulcers (DFUs) often result in severely adverse outcomes, such as serious infections, the need for hospitalization and lower extremity amputations, that are associated with a five-year mortality of about 50%[6-8].

Treatments for DFU include surgical debridement and drainage, antimicrobial therapy for infected wounds, pressure off-loading methods and advanced wound dressings. Despite these treatments, lower extremity amputations in diabetic patients occur at a rate 17 to 40-fold higher than in non-diabetic individuals[9]. The most frequent precipitating cause for non-traumatic foot amputations worldwide is a DFU with secondary infection[10-13]. Today, it is estimated that worldwide a foot is lost due to complications of diabetes every 30 s[6]. Thus, many clinicians and researchers have made efforts to develop adjunctive or complementary treatments to improve the outcome of DFUs. This paper presents a review of the epidemiology, pathogenesis and clinical manifestations of DFUs, and a discussion of the data available on relevant physical therapies and rehabilitation methods.

Epidemiology

While it is difficult to obtain firm data on the prevalence and incidence of diabetic foot ulcers, published trials suggest that approximately 15% of diabetic patients will develop a foot ulcer complication during their lifetime[14]. In one study in 37 primary care centers in the United Kingdom 5.3% of 811 type 2 diabetic patient were reported to have a diabetic ulcer at some point in their life[15]. In the United States, while the rate of hospitalization for diabetic foot ulcer was 5.4 per 100 diabetic patients in 1980, this figure increased to 6.9 in 2003[16].

The review of patients with diabetic foot ulcers shows that there are more male patients than female patients[17]. One retrospective review from Turkey of 142 cases found that 65% were males[18]. Diabetic foot ulcers occur in both in the type 1 and type 2 forms of diabetes, but there are far more people with type 2 disease. The average age of patients with a diabetic foot ulcer is about 60 years. The development of diabetic foot ulcer is more strongly associated with the duration of the disease rather than the age at disease onset[2].

Pathophysiology

Several factors are involved in the development of DFUs but the two main causes can be divided into primary and secondary pathologies. Primary etiologies involve peripheral neuropathy and vasculopathy, while secondary causes relate to complications from hyperglycemia[19]. Diabetic foot ulcers may be completely neuropathic (35%), completely ischemic (15%) or a mixed neuro-ischemic (50%)[6]. Injury to neural cells related to poorly controlled blood sugar levels cause motor, sensory and autonomic neuropathy, leading to altered foot anatomy (such as claw toe and hammer toe deformity), impaired sensory perception and formation of skin cracks[10,20-22]. Microvascular dysfunction and macrovascular (atherosclerotic) disease leads to ischemia through impaired blood supply to the feet (Figure 1). Ulcers usually occur in the feet due to physical, thermal or chemical trauma.

Musculoskeletal complications involving the foot, most commonly seen in patients with a longstanding history of diabetes, put patients at risk for developing ulcers. Major underlying causes include increased glycosylation of collagen in the skin and periarticular tissue (tendon, ligaments and the joint capsule), decreased collagen degradation, and diabetic microangiopathy[23]. Alterations in the structure of the foot, including loss of flexibility and limited joint mobility, impair the ability of the foot to absorb and redistribute forces related to impact with the ground while walking. The development of foot deformities is a major factor in creasing plantar pedal pressure. Among the foot joints, the metatarsophalangeal and subtalar joints are exposed to the highest level of pressure[23], which lead to the development of foot ulceration[14]. The factors discussed in this section are summarized in Table 1[10].

CLINICAL PRESENTATION

Classification

When assessing a patient with a diabetic foot wound, formulating a treatment approach requires understanding how to characterize any foot infection present as well as the patient's overall status. While there are now many schemes for assessment of diabetic foot pathology, the most commonly used classifications include those by Wagner, the University of Texas, and the International Working Group on the Diabetic Foot PEDIS system (the "infection" part of which is very similar to that proposed by the Infectious Diseases Society of America)[24-26]. While the Wagner scheme can predict clinical outcome, it is mainly useful for vascular and surgical classification and not for assessing infection[27]. The University of Texas classification is now more widely used, but it classifies infection only as present (which it does not define) or absent. On the other hand, the PEDIS classification is a research tool for assessing for circulatory impairment (perfusion), the extent and depth of the ulcer and neuropathy (sensation) as well as for the presence and severity of infection[25].

Assessment of the foot

Evaluating a patient with a diabetic foot wound requires assessing the dermatologic, vascular, neurologic and

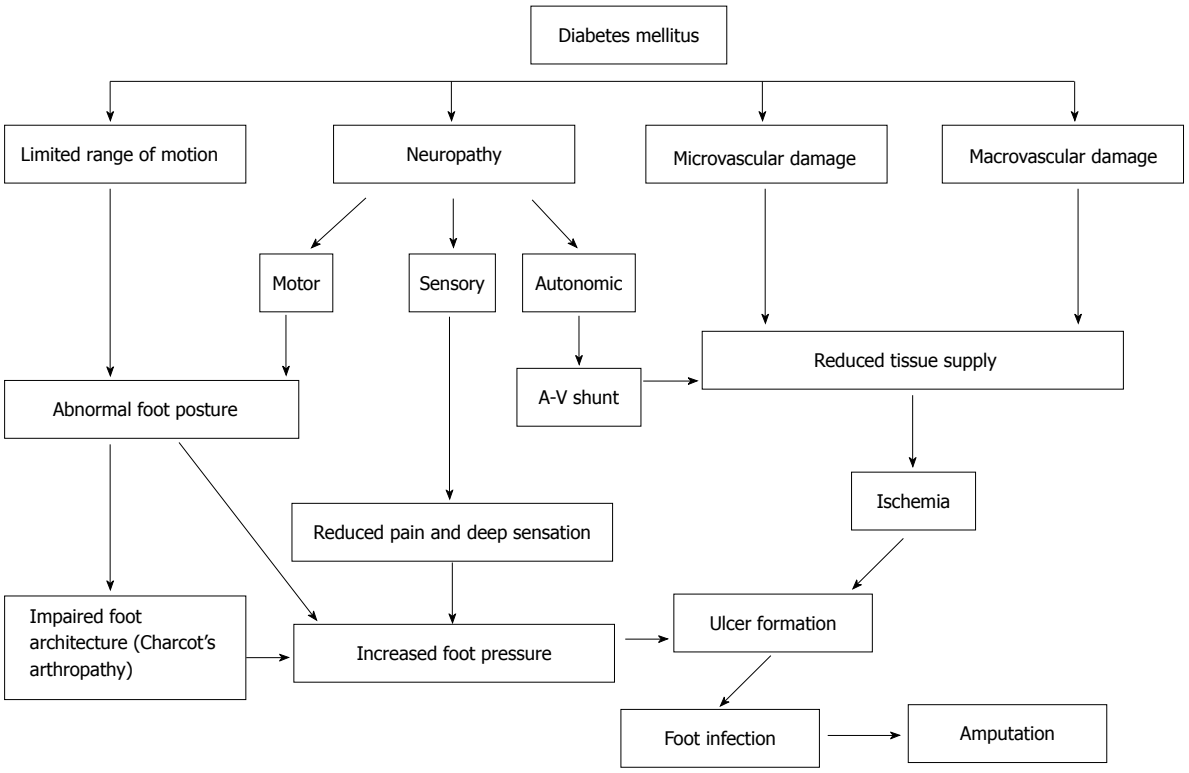

Figure 1 Diabetic foot pathogenesis.

Table 1 Causes and effects of the factors that lead to the diabetic foot infection pathogenesis[10]

Risk factor	Cause/effect of the risk factor
Peripheral motor neuropathy	Abnormal foot anatomy and bio-mechanics, manifesting with paw feet, high foot arch, subluxed metatarsophalangeal joints, increased foot pressure and callus formation
Peripheral sensory neuropathy	Minor chronic injuries secondary to heat, mechanical or high pressure as a result of deficiency of protective pain sensation
Peripheral autonomic neuropathy	Cracks on the dry skin due to reduced moisture
Neuroosteoarthropodic deformities	Abnormal foot anatomy and bio-mechanics secondary to increased foot pressure (particularly in the midplantar region)
Vascular failure	Reduced neutrophil migration, loss of the tissue viability and delayed wound healing
Uncontrolled blood sugar and other metabolic imbalances	Deficient immune system (particularly in the neutrophil functions), wound healing and collagen production
Patient characteristics	Loss of vision, limited motion, previous amputation(s)
Incompliant patient attitude	incompliance with the hygiene rules, foot care, prophylactic measures and healthcare principles (excessive weight gain, *etc.*)
Inadequacy of the healthcare system	Inadequate patient training on foot care, blood sugar control *etc.*; insufficient treatment centers that provide one-to-one patient care and/or insufficient bed counts; deficiency of a multi-disciplinary approach

musculoskeletal findings[2]. Important findings include any redness, swelling, increased warmth, pain or tenderness, numbness, skin breaks, blisters, peeling, ingrown toenails or other nail deformity, callus, or skin dryness[28]. The clinician should examine the dorsal, plantar, medial, lateral and posterior surfaces of the feet, along with the nails, comparing them to the upper extremity[28]. Assessment for peripheral vascular disease includes palpating the dorsalis pedis, tibialis posterior, popliteal and superficial femoral arteries checking the skin for paleness and coolness compared to more proximal regions of the lower extremity[28].

While the probability of having peripheral neuropathy is 30%-40% in patients with a 10-year diabetes history, and up to 60%-70% of those with a diabetes over 25 years, the rate is over 80% in those with a foot ulcer[14]. The neuropathy is a distal, symmetrical, sensorimotor type that may be asymptomatic or manifest with pain, allodynia, numbness or a burning sensation[29]. Because the small diameter fibers are involved early, pain and heat sensation disappear first. Subsequently, with large-diameter motor neuron damage there is a reduction in deep tendon reflexes, vibration and pressure sensation[2]. The vibration sensation can most easily be measured with a tuning fork, with the normal time of vibration perception approximately 20 s.

Table 2 Physical therapy modalities potentially beneficial for wound healing

Heating agents	Electrotherapy methods
Superficial heaters: Infrared treatment, global heat treatment	Electrical stimulation
Deep heaters: Ultrasound treatment	Shock wave therapy (ESWT)
	Laser treatment
	Magnetic field treatment
	Galvanic current treatment

ESWT: Extracorporeal shock wave therapy.

Superficial sensation can be examined by touching with cotton, two point discrimination by using the blunted end of a compass placed on the dorsal foot. The most commonly used and well-validated test is checking for presence of pressure sensation with a monofilament. Absence of the ability to feel a 5.07-size monofilament at selected sites on the foot means the patient lacks protective sensation, which greatly increasing the risk of a diabetic foot ulcer[29]. Foot deformities related to motor neuropathy include pes cavus, pes planus, hallux valgus, hallux malleus, claw toe, and Charcot's arthropathy[23]. This impairs the architecture of the foot leads to loading excessive pressure onto certain points, increasing the risk of ulceration.

PHYSICAL THERAPY AND REHABILITATION METHODS

Outcomes have consistently been shown to be better when patients with a diabetic foot ulcer are cared for a multi- (or, more accurately, inter-) disciplinary approach. Disciplines involved should optimally include a diabetologist, infectious diseases/microbiology expert, plastic, orthopedic or podiatric surgeon, angiologist or vascular surgeon, physical therapist, wound care nurse, orthotist and rehabilitation expert. The treatment plan should be established by joint council, optimally with as many team members in the same room with the patient as possible.

Diabetic foot ulcers prolong the duration of hospitalization to a greater extent than almost all other diabetes-related complications[30]. And, despite great advances in the treatment of diabetic foot ulcers, a substantial minority of patients still undergoes a lower extremity amputation. In fact, complications of diabetes are the leading cause of non-traumatic lower leg amputations worldwide. These amputations are associated with major morbidity, worsening of quality of life and financial costs, but perhaps most importantly, the five year post-amputation patient survival is only 50%. Therefore, every means possible should be used to try to heal a diabetic foot ulcer, including exercise therapy, footwear modifications physical therapy and rehabilitation methods. Currently, there is some published literature on the value of physical therapy and rehabilitation methods for diabetic foot ulcers, but no Cochrane Database of Systematic Reviews, National Guidelines or consensus declarations.

Physical therapy modalities

The physical treatment modalities used in diabetic polyneuropathy and diabetic foot ulcers are summarized in Table 2.

Thermotherapy: Heat, a form of energy, is a commonly used physical treatment agent. When a hot substance contacts a cold one, it transfers of heat. During superficial heat treatment the heated therapeutic agents transfer heat to the body[31].

Heat treatment increases blood flow to the area as a result of inducing vasodilatation, which is thought to contributes to wound healing[31]. There is, however, only one study evaluating the benefits of this treatment for chronic wounds in persons[32]. In this study, patients who had previously received electrical stimulation were treated with either global heat treatment or local heat treatment. Global heat treatment was administered by keeping the patients in rooms at 32 ℃ of temperature for 20 min after establishing a heater and a fan system in a room. Local therapy was administered using an infrared heat lamp heated to 37 ℃ that was directed on the wound site for 20 min. The control subjects only received treatment with electric stimulation, which was administered at 30-Hz frequency and 20-mA ampere with 2-cm × 2-cm-diameter carbonized electrodes. Wound healing was calculated by measuring the length of the wound with a digital machine, the depth with a metric ruler and the blood flow with laser Doppler ultrasound. Wound healing rates were significantly higher (by 20%) with global heat treatment compared to the local heat therapy. With local heat therapy the blood flow measured by laser Doppler ultrasound had increased, but it was relatively lower than that in global heat treatment. Wound healing in the control (electric stimulation) group was less than in the two heat treated groups[32]. Although local heat therapy is more practical to administer, global heat treatment was superior for improving wound healing.

Sound waves turn into heat energy while passing through a homogenous environment. Ultrasound involves sound waves with a frequency higher than what the human can hear. Deep-heating agents such as ultrasound can effectively heat the skin as deep as > 1 cm. The sound frequencies used in ultrasound therapy are typically 1.0 to 3.0 MHz (1 MHz = 1 million cycles per second) at amplitude densities of 0.1 to 3 w/cm^2[33] (see Figure 2). The presumed modes of action of ultrasound include causing vasodilatation, increasing cell metabolism, enhancing cell permeability, inducing fibroblast proliferation, releasing vascular endothelial growth factor, increasing collagen's flexibility, and reducing edema by increasing the interstitial fluid flow. The result of all these effects is to improve wound healing[33].

Contraindications to ultrasound treatment include

Figure 2 Ultrasound treatments administered following diabetic foot ulcer formation.

directing acoustic energy over a malignant lesion, pregnant abdomen, or a plastic or metal implant. Additionally, this therapeutic mode should not be used in patients with decompensated cardiac failure, cardiac pacemaker, acute infection, hemorrhagic diathesis[33]. Ultrasound treatments are categorized by their frequency, intensity, pulsed or continuous administration, and tissue-contact or contact-free administration[34]. When ultrasound is used for debridement it creates cavitation effects in the tissue; at high doses this may result in a level of cavitation that is not stable. Low-frequency contact, or contact-free ultrasound treatment, activates the cell membrane and induction of fibroblast proliferation with DNA synthesis, elicitation of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) in osteoblasts[35]. When ultrasound therapy is used in musculoskeletal diseases at low frequencies (such as 20-40 kHz) its thermal effect is reduced and the debridement effect becomes more prominent; concomitant bactericidal and wound healing effects have also been observed[34].

Ennis *et al*[36] conducted a multicenter, double-blind randomized controlled trial that compared active 40-Khz ultrasound treatment to sham ultrasound treatment in 3 sessions per week for 12 wk in patients with a chronic diabetic foot ulcer. In both groups the rates of wound healing were increased after treatment compared to pre-treatment rates. In neither group was there any statistically significant improvement in wound exudation or wound closure.

A newly discovered ultrasound modality called contact-free low-frequency ultrasound is thought to improve wound healing by inducing fibroblast proliferation and releasing VEGF and IL-8, thereby reducing growth of bacterial in chronic wounds[37]. In a study performed by Yao *et al*[37], 12 patients were grouped into those who received: (1) contact-free low-frequency ultrasound at 40 kHz three times a week; (2) contact-free low-frequency ultrasound at 40 kHz once a week, both in addition to standard treatment; and (3) only standard treatment for 4 wk. The group receiving contact-free low-frequency ultrasound three times a week had significantly higher rates of reduction in the size of the wound than the two other groups, while there was no significant difference between the second and the third groups. Thus, contact-free low-frequency ultrasound administered three times a week appeared to be most effective in reducing the area of the wound[37].

Voight *et al*[34] conducted a systematic review and meta-analysis to examine studies of low-frequency (20-30 kHz) ultrasound delivered at either low or high intensity. They found 8 randomized controlled trials, in five of which both groups received standard treatment modalities (debridement, wound healing) but only one group also received active to low-intensity contact-free ultrasound, while the control group received sham ultrasound therapy. In the other three trials either low-frequency or high-intensity contact therapeutic ultrasound treatment was administered. The results of these trials demonstrated that early healing (at $\leqslant$ 5 mo) in patients with diabetic foot ulcers was favorably influenced by both high- and low- intensity ultrasound delivered at a low frequency, either *via* contact or noncontact techniques. The authors concluded that low-frequency, low-intensity, noncontact ultrasound is more effective at producing complete healing than standard wound care[34].

Electrotherapy methods: Electrical stimulation is normally used in physical therapy to strengthen paralytic muscles, but there are also clinical trials that suggest it is beneficial in treating diabetic foot ulcers. Electrical stimulation wound therapy produces short pulse electrical stimuli intended to mimic the body's natural electrical system and stimulate wound repair[38]. The mechanism of action appears to involve an effect on calcium channels in the cell membrane that increases the intra-cellular calcium permeability, which stimulates the production of nitric oxide (NO) by increasing the nitric oxide synthase (NOS). NO, a strong vasodilator enhances the blood flow, thereby potentially accelerating wound healing. It also can change form to create peroxynitrate which has strong bactericidal effects. In addition, NO enhances the transfer of glucose into cells and increases epithelization and collagen storage[39]. Electrical stimulation may also stimulate the migration of various wound-modifying cells including keratinocytes, fibroblasts, macrophages, and neutrophils *via* various signaling mechanisms[40]. Electrical stimulation is typically administered at 30-Hz frequency, at a pulse every 250 microseconds, and 20-milliampere current, using 5 cm × 5 cm disposable carbonized electrodes, for 30 min three times weekly[41]. This treatment modality should not be used in patients with a cardiac pacemaker[39].

Decreased local tissue perfusion and the subsequent tissue hypoxia contribute to the occurrence, and failure to heal, of a foot ulcer in many diabetic patients[6]. Some clinical trials suggest that the tissue perfusion in chronic ulcers is increased following electrical

stimulation and this is associated with wound healing[42]. One evaluation following electrical stimulation, using laser Doppler flowmetry, demonstrated a significant increase in tissue perfusion achieved in patients with diabetes and peripheral vascular disease[43]. In another trial, the transcutaneous oxygen pressure levels were significantly increased within the first 5 min following electrical stimulation in diabetic patients with peripheral vascular disease[44].

Studies in the literature used different protocols of electrical stimulation for diabetic foot ulcers since. In two, treatment with electrical stimulation was compared to sham treatment[45,46] while in another two studies electrical stimulation was compared to infrared heat lamp[32,41]. Another study compared electrical stimulation to two different control groups, one receiving electrical current at a very low dose (4-mA intensity) and the other not receiving electrical stimulation[47]. While all these studies investigated the effect of electrical stimulation the authors did not provide any data on calculation of the sample size and streaming[41,45,46] and the electrical current was administered as symmetrical biphasic, monophasic or square wave current. In all 5 trials the rates of healing, measured by the diameter of the wound, were the main result; in 4 the rates of healing were significantly higher in the study group compared to the control group[32,41,45-47]. Another randomized study by Peters *et al*[46] enrolled a total of 40 patients with diabetic foot ulcer. Twenty patients were randomized into each of the control group and the study group. The study group received electrical current *via* a micro-computer each night for 8 h, while the control group received no current. The patients were followed up for 12 wk, or until at least one patient achieved healing, and the healing rates were not significantly different for the two groups.

Extracorporeal shock wave therapy (ESWT) is a non-invasive method of treating certain soft tissue injuries. It focuses strong sound waves on affected site using an ellipsoid-shaped steel probe. This device can administer an amount of energy 10-fold higher than delivered by ultrasonic devices within 1 microsecond. While ESWT is non-invasive, it delivers high-amplitude, short, single, pulsatile, acoustic waves that distribute their mechanical energy into the environment while passing from the soft tissue to the bone. If the delivery is < 0.1 mJ/mm^2 it is classified as low-energy, if 0.1-0.2 mJ/mm^2 it is middle-energy and if > 0.2 mJ/mm^2 it is high-energy. Recommendations for the treatment of diabetic foot ulcer are generally administration at an energy level of 0.03 mjoul/mm^2 twice weekly for a total of 6 applications, to achieve 100 pulses/cm^2. The treatment may take up to 30 min per foot[48].

ESWT has been extensively studied over the past 20 years and been found to be effective for the treatment of various musculoskeletal diseases (plantar fasciitis, calcific tendonitis of the shoulder, tennis elbow, pseudo-arthrosis and patellar tendinitis). The mechanism of action involves the stimulation of tissue healing, reduction of calcifications and inhibition of the pain receptors[49]. It appears to induce the early expression of angiogenesis-associated growth factors, endothelial NO synthetase and vascular endothelial growth factors, thus increasing cell proliferation and accelerating tissue regeneration and healing[50]. Our literature search identified two clinical trials investigating ESWT for foot ulcers. One enrolled 30 patients with diabetic foot ulcer, all of whom received standard treatment (wound debridement, infection treatment, adequate pressure transfer), but the study group also received ESWT. They found significantly better results in complete wound closure, healing time, and re-epithelization indices following a 20 wk of treatment in the ESWT group[48]. In another trial, 32 wound in 30 patients who were unresponsive to conservative or advanced dressing treatment received ESWT, while control group of 10 patients received standard treatment (wound debridement, infection treatment and adequate pressure transfer). Complete wound closure was achieved after 6 sessions of ESWT in 16 patients the study group. In wounds with incomplete healing exudate was reduced, granulation tissue increased and the size of the wound decreased significantly after 4-6 sessions of ESWT. The study group also had a significant reduction in pain and a significant increase in wound healing compared to the control group[51]. These limited results suggest that ESWT may be beneficial and safe in the treatment of diabetic foot ulcers. This modality is contraindicated in patients with previous cardiac bypass operation, active pregnancy, major cancer or coagulation disorder.

There are three types of laser treatment, based on their potency (see Table 3)[52]. At physical or occupational therapy units low level lasers (typically between 5 mW to 500 mW of output power) are typically used in treatment. Low-level laser light has been reported to be effective in the treatment of impaired microcirculation (thought to be a relatively common problem in patients with a diabetic foot ulcer), wound healing and pain syndromes. The mechanism of action is thought to involve enhancement of the blood circulation and stimulation of the neo-angiogenesis through an increase in skin heat[52].

In a double-blind, placebo-controlled clinical trial in patients with diabetic microangiopathy, 15 received low-level laser therapy at a dose of 30 J/cm^2, while 15 patients received a sham beam[53]. The results revealed a significant increase in the skin heat 15 min, following laser treatment compared to the control group. Presumably this was related to the increased skin blood circulation and contributed to improved healing[53]. In another randomized controlled trial of patients with a diabetic foot ulcer, 13 received low-level laser therapy and 10 patients were in a placebo group[54]. Wound size was significantly reduced at week 4 in the low-level laser therapy compared with the placebo group, and at week 20 eight patients in the

Table 3 Laser types used in the clinical practice and their contents

Low-level lasers (cold laser)	Helium and neon
Moderate-level lasers	Gallium, aluminum-arsenide
Potent lasers	Argon-CO-Yttrium aluminum oxide

treatment group and only three in the placebo group achieved complete wound healing. The mean duration until complete wound healing was not statistically significant: week 11 in the treatment group compared to week 14 in the control group[54]. These limited data suggest there may be a role for low-level laser therapy in accelerating healing of diabetic foot ulcers wound.

Magnetic field treatment use magnets or magnetism. Magnetotherapy is believed to have positive impact on immunological condition of the patient and causes dilation of blood vessels secondary to changes in autonomic nervous system, thereby removing toxins causing pain. Additionally, it is may increase the permeability of neuronal membranes and promoting release of hormones with analgesic action such as endorphin[55].

For the treatment of a diabetic foot ulcer, magnetic field treatment is generally administered for one hour daily for 10 d, at low intensity (30 Gauss). Using this magnetic field device, the foot is placed in a solenoid coil. During the procedure magnetic field depth of penetration is 20 mm[56].

Another trial investigating the efficacy of magnetic field treatment randomized 375 diabetic patients with symptomatic polyneuropathy to either wearing 450 G magnetic insoles (study group) or non-magnetic insoles (control group) for 4 mo. The study group had a greater reduction than the control group in pain, numbness and burning and this improvement was maintained for 4 mo[56].

The limited published data suggest that magnetic field treatment may be effective modality in reducing symptoms of diabetic polyneuropathy. This physical therapy modality is contraindicated in patients with pregnancy, implanted inner ear haring device and other small metallic implants[55].

Galvanic (direct) current treatment uses zero-frequency electrical current. The current flows in one direction and has polarity current. Through stimulation of the myelin-free pain fibers, this current achieves paresthesia of both the superficial and the deep skin layers[57]. It is administered by carbon silicon surface electrodes with a current intensity of 1 Ma for duration of 20 min[57] in the treatment of a diabetic foot ulcer (Figure 3).

To our knowledge, there is only one published study in the literature that investigated the efficacy of treatment of diabetic ulcer wounds with galvanic stimulation. In this study 11 diabetic patients with impaired peripheral perfusion pressure (transcutaneous oxygen tension of < 40 mmHg) received galvanic stimulation treatment for 60 min on each of 2 d.

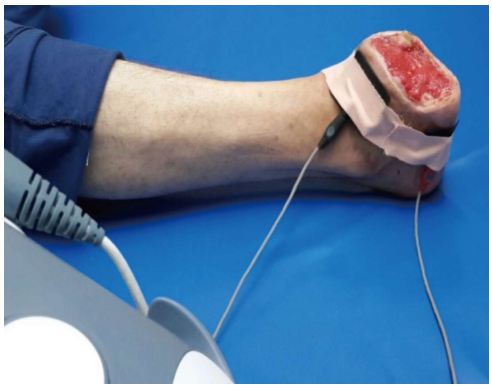

Figure 3 Galvanic current administered following diabetic foot ulcer formation.

Measurements were then performed of transcutaneous oxygen in the dorsum of both feet *via* and of skin blood flow *via* laser Doppler flowmetry. The authors reported a significant increase in perfusion in the patients who received galvanic stimulation within the first 5 min, compared to the control group[44]. This modality is contraindicated in pregnant women and in patients with cardiac pacemaker[57].

Exercise treatment: Recent studies suggested that exercise, may be an effective therapeutic modality for patients with a diabetic foot ulcer. Range of joint motion, stretching exercises, Buerger-Allen exercise, and proprioception and balance exercises may be helpful in patients with, or predisposition to, a diabetic foot ulcer[58-60]. Exercises involving range of joint motion or stretching in all directions may increase the blood flow to the feet. Proprioception exercises increase the sensory input in patients with diabetic polyneuropathy, thereby enhancing their perception capacity and ability to protect the extremity. These exercises may lead to a reduced risk of falling related to improved balance and coordination. In addition, as well as these exercises, balance and coordination exercises may lead to a reduced risk of falling related to improved.

Using the Buerger-Allen exercises may enhance the blood supply to the extremity, potentially leading to formation of new vascular structures. While performing this exercise the patient should lie in supine position for 3 min, lifting his/her feet to a higher ground. Then, he/she should sit and keep both feet in the following positions for three minutes each: flexion, extension, pronation and supination. The feet should turn pink (related to improved blood flow) upon practicing these movements; if they become blue or painful, the patient should lift his/her feet to a higher ground again and rest, as needed. At the end of the exercise, the patient should lie in a supine position for 5 min, keeping the feet warm by wrapping them up with a blanket.

In a study by Goldsmith *et al*[60] investigating the efficacy of range-of-motion exercises in diabetic foot patients, they asked the patients to draw letters

of the alphabet with their feet and also practiced passive and active dorsiflexion of the ankles and metatarsophalangeal joints, plantar flexion, active subtalar joint pronation and supination, stretching of the gastrocnemius and soleus muscles. The authors reported that these exercises resulted in a reduction in the joint limitation and foot plantar pressure during walking[60]. In patients with a diabetic foot ulcer, Flahr *et al*[59] assessed the effects of 10-repetition foot exercises, consisting of the active inversion, eversion, dorsiflexion and plantar flexion of the feet and ankles, practiced twice daily. In this prospective, quasi-experimental pilot study, following a 12-wk exercise program the patients practicing the exercise were reported to have faster ulcer healing compared to those who did not participate in the exercises. The mechanism of action of these foot exercise is believed to involve increase of the blood supply to the region, the resulting in improved wound healing[59].

Shoe modification: Wearing inappropriate (*i.e.,* poorly fitting) shoes may lead to formation of callus, redness, blisters and eventually various deformities. As diabetic patients, especially those with peripheral neuropathy or foot deformity, are especially prone to these problems they may need appropriate shoe modifications to avoid foot ulcers. Such patients should use specially manufactured shoes with large and high finger toe box and rocker bars, made from soft and flexible leather. Shoes with rocker bars reduce the ground reaction force and facilitate the push-off phase of walking. Rigid insoles should be avoided[61] Using plastazote insoles may help ensure a homogenous distribution of the load. The heels of the shoe should be supported with a soft pad and made from at least two materials of different densities, with a robust edge and a capacity to absorb light shocks. The soles should be renewed every 6 to 12 mo.

Prophylactic methods for preventing foot ulcers development: After a foot ulcer, the rate of recurrent ulceration has been reported to be 28% in the first year, reaching up to 100% at 40 mo in diabetic patients ulcer is the precursor in more than 85% of foot amputations[61]. Thus, it is crucial to apply prophylactic methods to these high risk patients. Every professional who treats patients with diabetes must receive continuing professional education and clinicians should provide regular foot examination for diabetic patients. Patients should also be informed about their disease, the potential related foot complications, and the importance of foot inspection and care and glycemic control. In addition to regular professional foot examinations patients should be instructed to check their feet every day (including with a mirror to see the bottoms) for the presence of skin breaks, redness, swelling, callus or other problems. The patient should also be instructed to wash the feet with warm water (checking the temperature with the hand, not the foot) and to dry them with a soft towel every day, then apply an appropriate moisturizer. The nails should be cut carefully with a good quality nipper, with a straight border; this may need to be done by a medical professional and patients should avoid pedicures. Many patients view their own homes as "safe", but they should be advised that there, and elsewhere, they should avoid walking on bare foot, or using flimsy or poorly fitting sandals or slippers. It is best to alternate footwear during the week to avoid excess pressure in specific areas and to allow the perspiration in the shoes and insoles to evaporate. The patients socks should be made from cotton and be seam-free. They also should be warned against smoking to avoid worsening blood circulation[62].

CONCLUSION

In summary, the treatment of diabetic foot wounds requires a multidisciplinary approach. It may include physical therapy and rehabilitation methods. Unfortunately, studies on this topic were conducted with moderate quality of evidence. It is required to be supported by larger randomized trials.

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ORIGINAL ARTICLE

Clinical Trials Study

Barriers in contribution of human mesenchymal stem cells to murine muscle regeneration

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Abstract

AIM: To study regeneration of damaged human and murine muscle implants and the contribution of added xenogeneic mesenchymal stem cells (MSCs).

METHODS: Minced human or mouse skeletal muscle tissues were implanted together with human or mouse MSCs subcutaneously on the back of non-obese diabetic/severe combined immunodeficient mice. The muscle tissues (both human and murine) were minced with scalpels into small pieces (< 1 mm^3) and aliquoted in portions of 200 mm^3. These portions were either cryopreserved in 10% dimethylsulfoxide or freshly implanted. Syngeneic or xenogeneic MSCs were added to the minced muscles directly before implantation. Implants were collected at 7, 14, 30 or 45 d after transplantation and processed for (immuno)histological analysis. The progression of muscle regeneration was assessed using a standard histological staining (hematoxylin-phloxin-saffron). Antibodies recognizing Pax7 and von Willebrand factor were used to detect the presence of satellite cells and blood vessels, respectively. To enable detection of the bone marrow-derived MSCs or their derivatives we used MSCs previously transduced with lentiviral vectors expressing a cytoplasmic *LacZ* gene. X-gal staining of the fixed tissues was used to detect β-galactosidase-positive cells and myofibers.

RESULTS: Myoregeneration in implants of fresh murine muscle was evident as early as day 7, and progressed with time to occupy 50% to 70% of the implants. Regeneration of fresh human muscle was slower. These observations of fresh muscle implants were in contrast to the regeneration of cryopreserved murine muscle that proceeded similarly to that of fresh tissue except for day 45 (P < 0.05). Cryopreserved human muscle showed minimal regeneration, suggesting that the freezing

procedure was detrimental to human satellite cells. In fresh and cryopreserved mouse muscle supplemented with LacZ-tagged mouse MSCs, β-galactosidase-positive myofibers were identified early after grafting at the well-vascularized periphery of the implants. The contribution of human MSCs to murine myofiber formation was, however, restricted to the cryopreserved mouse muscle implants. This suggests that fresh murine muscle tissue provides a suboptimal environment for maintenance of human MSCs. A detailed analysis of the histological sections of the various muscle implants revealed the presence of cellular structures with a deviating morphology. Additional stainings with alizarin red and alcian blue showed myofiber calcification in 50 of 66 human muscle implants, and encapsulated cartilage in 10 of 81 of murine muscle implants, respectively.

CONCLUSION: In mouse models the engagement of human MSCs in myoregeneration might be underestimated. Furthermore, our model permits the dissection of species-specific factors in the microenvironment.

Key words: Skeletal muscle; Muscle regeneration; Muscle implants; Mesenchymal stem cells; Satellite cells

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Core tip: The translational relevance of animal models for tissue repair is often ambiguous. We describe here a murine model for the comparison of the regeneration of damaged human and murine skeletal muscle implants and the contribution of human and mouse mesenchymal stem cells (MSCs) to this process. Our findings suggest that murine muscle tissue provides a suboptimal environment for maintenance of human MSC, and that in mouse models their capacity to engage in myoregeneration is underestimated. The added value of the present model is that it permits the dissection of species-specific factors in the microenvironment.

de la Garza-Rodea AS, Boersma H, Dambrot C, de Vries AAF, van Bekkum DW, Knaän-Shanzer S. Barriers in contribution of human mesenchymal stem cells to murine muscle regeneration. *World J Exp Med* 2015; 5(2): 140-153 Available from: URL: http://www.wjgnet.com/2220-315X/full/v5/i2/140.htm DOI: http://dx.doi.org/10.5493/wjem.v5.i2.140

INTRODUCTION

The recent advances in (1) the derivation of human pluripotent stem cells; (2) the characterization and *ex vivo* amplification of human somatic stem cells; and (3) the genetic modification of these cells have created new prospects for cell-based therapies. The therapeutic potential of (engineered) human stem cells should ideally be validated in humans. Due to practical and ethical restrictions this type of study is, however, largely restricted to animals.

After transplantation of different human stem cell types including pericytes[1], satellite cells[1], mesenchymal stem cells (MSCs)[2] and muscle precursor cells[3] into damaged murine skeletal muscle, typically 1%-7% of the myofibers in the regenerated tissue contained human nuclei. Similar experiments performed with allogeneic satellite cells injected into muscles of mdx mice[4] (a mouse model for Duchenne muscular dystrophy) showed more than 10% chimeric myofibers after the administration of a significantly smaller cell dose than was used for the xenotransplantation studies. The reconstitution frequency by syngeneic donor cells was even more profound in mdx mice transplanted with a subpopulation of satellite cells with 94% of all myofibers becoming chimeric[5]. Although these findings require confirmation by direct comparative studies, they suggest a higher propensity of murine than of human (stem) cells to participate in the regeneration of mouse skeletal muscle tissue. Consequently, the results of preclinical studies with human stem cells in mice may lead to an underestimation of their therapeutic potential in humans.

The present study is an attempt to develop a method for investigating this assumption. This method is based on the free grafting together with human MSCs (hMSCs) or mouse MSCs (mMSCs) of minced human or mouse skeletal muscle implanted under the subcutis of mice. The reason to work with minced tissue was that it permits an even distribution of added MSCs throughout the implant. Successful free grafting of mammalian muscles was first accomplished in the 1960s[6]. As implants, either intact or minced skeletal muscle pieces have been used. Transplantation of these materials occurred into an emptied skeletal muscle bed or at a heterotopic anatomical site[7-10]. Under all conditions, myoregeneration was preceded by host-mediated vascularization and innervation[6,11] of the grafted tissue. We selected the subcutis as the site of implantation to preclude participation of host skeletal muscle cells in the regeneration of the graft[7,8,10]. The study includes both human and murine muscle grafts, both fresh and cryopreseved, supplemented with either mouse- or human bone marrow (BM)-derived MSCs. Non-obese diabetic/severe combined immunodeficient non-obese diabetic (NOD)/LtSz-scid/scid/J [in brief NOD/severe combined immunodeficient (SCID)] mice served as hosts.

MATERIALS AND METHODS

Skeletal muscle tissues

Human skeletal muscle specimens were collected from anonymous surgical "waste" material in orthopedic surgery. In agreement with the pertinent Leiden University Medical Center (LUMC; Leiden, the Netherlands) guidelines, and in accordance with the Best Practices code of Dutch Federation of Biomedical Scientific Societies, and the

based on article 467 of the "Wet op de Geneeskundige Behandelingsovereenkomst (WGBO)" no informed consent is required for the use of anonymous and non-traceable body materials and the institutional ethics committee of the LUMC waived the need for patient consent. The samples (from 53 patients, 26 females and 27 males, in an age-range of 26 to 82 years) were washed once with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO), freed of tendons and clumps of non-muscle tissue and chopped with scalpels into fragments < 1 mm^3.

Mouse skeletal muscle tissue was collected from all legs, dorsum and abdomen of 2 male- and 8 female-BALB/c (2 to 21 mo old) mice or 1 male-C57BL/6 (8 mo old) mouse (Harlan, Venray, the Netherlands), pooled and chopped with scalpels into fragments measuring < 1 mm^3.

Both human and mouse muscle mince were aliquoted in portions of 200 mm^3. These were either cryopreserved or freshly implanted within 3 h after collection.

The minced tissue aliquots used for preservation were suspended in ice-cold culture medium (see next section) supplemented with 10% dimethylsulfoxide (DMSO; Sigma-Aldrich), kept at -80 ℃ overnight and subsequently stored in liquid nitrogen vapor until use. Prior to implantation, the tissues were thawed and DMSO was removed by washing 3 times with PBS.

Isolation and culture of MSCs

hMSCs were isolated from BM of "waste" material collected according to the guidelines of the LUMC (mentioned above) during orthopedic surgery performed on a 38-year-old-female. Cells were isolated and cultured as previously described[12]. Cell expansion was performed in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, L-glutamine, sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS; all from Invitrogen, Breda, the Netherlands) and 0.5 ng/mL basic fibroblast growth factor (FGF2; Sigma-Aldrich) in CELLSTAR cell culture flasks (Greiner Bio-One, Frickenhausen, Germany) at 37 ℃ in humidified air containing 10% CO_2.

mMSCs were isolated from BM of 16-wk-old female-BALB/c mice and cultured under the same conditions as the hMSCs.

hMSCs at passage number 4 and mMSCs at passage number 6 were transduced with LV.EF1a.CMV.LacZ as previously described[2].

The tumorigenic potential of mMSCs of passage 14 was tested through subcutaneous injection of 10^6 cells in 2 male NOD/SCID of 8-wk-old. Animals sacrificed at 15 and 36 d after transplantation did not show any macroscopic alterations in primary organs and did not display abnormally growing cell masses at the site of injection (data not shown).

Animals and subcutaneous implants

Recipient mice for the human and BALB/c mouse muscle mince were NOD/SCID mice from a breeding colony established with animals from Jackson Laboratory (Bar Harbor, ME). In total 70 mice were used (43 females and 27 males) with an age range of 2 to 13 mo. C57BL/6-Tg (CAG + EGFP) C14-Y01-FM131Osb[13] recipient mice (4 females of 4 to 5 mo) of which almost all tissues including skeletal muscle expressed a recombinant enhanced green fluorescent protein (*eGFP*) gene were used to detect contribution of host cells to the regeneration of the muscle implants. These mice were obtained from Dr. Masaru Okabe, Osaka University, Japan and received donor skeletal muscle tissue from C57BL/6 mice (described above). All mice were kept in the Animal Facility of the LUMC following the internal guidelines. Experimentation with animals was performed in compliance with a protocol approved by the animal ethics committee of the LUMC.

Minced muscles of either human or mouse origin were implanted subcutaneously on the back of the mouse. Routinely, each NOD/SCID mouse received two implants, one of human and one of murine origin, to minimize variability caused by recipient-associated conditions. Grafting was performed under aseptic conditions and general anesthesia with isoflurane. The back of the mouse was shaved and rinsed with ethanol. Next, two longitudinal 1-cm incisions to the left and right of the spine were made with a scalpel. The incisions were enlarged using scissors (Fine Science Tools, CA, United States) dissecting the skin from dorsal fascia thus forming a dermal pocket in which a standard volume of 200 mm^3 minced muscle tissue alone or thoroughly mixed with 5×10^5 MSCs was deposited. The MSCs were always freshly harvested from cultures in logarithmic growth phase. The wound was closed with two or three ETHICON PROLENE polypropylene size 5-0 sutures (Johnson and Johnson Medical, Amersfoort, the Netherlands). After 7, 14, 30 or 45 d mice were sacrificed by cervical dislocation, the implants were removed and processed for (immuno)histological analyses.

Tissue processing and (immuno)histochemistry

The excised implants were cut in two halves and fixed either overnight at 4 ℃ or for 1 h at room temperature in 4% buffered formaldehyde (Mallinckrodt Baker, Phillipsburg, NJ). Tissues fixed at room temperature were stained with X-gal (Sigma-Aldrich) as previously described[2].

All samples were embedded in paraffin, cut into 6-μm-thick sections and consecutive sections were placed on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) for histochemical and immunohistological staining.

Tissue sections of each sample were deparaffinized, rehydrated with graded ethanol-water mixtures and stained with hematoxylin (Klinipath BV, Duiven, Netherlands), phloxin (Sigma-Aldrich) and saffron (Ghohestan, Iran) (HPS) following standard procedures.

After dehydration, the sections were mounted with Pertex mounting medium (Histolab Products, Gothenburg, Sweden).

Direct microscopical screening of HPS-stained sections revealed in some implants areas reminiscent of bone and cartilage. Consecutive sections of those implants were stained with Alizarin Red S or Alcian Blue (both from Sigma-Aldrich) to visualize calcium deposits and sulfated glycosaminoglycans, respectively. Staining was performed on deparaffinized tissue sections, which were incubated for 2 min with Alizarin Red S or for 30 min with Alcian Blue. The Alcian Blue-stained sections were rinsed with water and counterstained for 5 min with Nuclear Fast Red (Sigma-Aldrich). Sections treated with either protocol were twice rinsed with water prior to dehydration and mounting on slides with Pertex mounting medium.

Immunohistology was used to assess myoregeneration as based on the presence of myoblast and/or multi-nucleated myofibers that stain positive for desmin; satellite cells that stain positive for Pax7; blood vessels detected by von Willebrand Factor (vWF) and LacZ labelled cells and their derivatives (beta Gal).

Tissue sections were deparaffinized, rehydrated and boiled for 10 min in 10 mmol/L citrate buffer (Sigma-Aldrich, pH = 6.0) for antigen retrieval. After rinsing the slides with water, endogenous peroxidase was inactivated by a 10-min incubation at room temperature with 0.3% (w/w) hydrogen peroxide (Sigma-Aldrich) in water. Following two additional washes with PBS a 1 h blocking step was performed using 10% normal goat serum (Dako Netherlands, Heverlee, Belgium) in PBS. Next, mouse monoclonal antibodies specific for desmin (clone D33; IgG1, κ; Dako Netherlands, dilution 1:100) or directed against chicken Pax7 (IgG1, κ; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; dilution 1:20) or the rabbit polyclonal anti-human vWF antiserum (that interacts with murine cells; Dako Netherlands; dilution 1:3000; also binds murine vWF) were added to sections for an overnight incubation at 4 ℃. The following day, the sections were washed in PBS and the secondary antibodies, either horseradish peroxidase-linked goat anti-mouse IgG (Dako Netherlands; dilution 1:100) or horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako Netherlands; dilution 1:100) were applied for 30 min. The binding of the antibodies was visualized with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich). The sections were counterstained with hematoxylin, dehydrated and mounted with Pertex mounting medium. Images were captured with a Color View IIIu camera mounted on an Olympus BH-2 microscope and processed using CELL^F imaging software (all from Olympus Nederland, Zoeterwoude, Netherlands).

For the detection of eGFP-positive satellite cells an eGFP-specific rabbit polyclonal antiserum was used together with the aforementioned murine anti-Pax7 monoclonal antibody. Deparaffinized and rehydrated tissue sections were immersed twice in 10 mmol/L citric acid solution (pH = 6.0) for 5 min at 90 ℃. Following cooling and washing steps the sections were blocked for 2 to 3 h with 4% IgG- and protease-free bovine serum albumin (Jackson ImmunoResearch Europe, Newmarket, United Kingdom) in PBS. Next, the sections were sequentially incubated with goat anti-mouse IgG (H + L) AffiniPure Fab fragment (MouseFab, Jackson ImmunoResearch; dilution 0.05 mg/mL) for 30 min, with the anti-Pax7 antibody overnight, with biotin-SP-conjugated AffiniPure goat anti-mouse IgG (H + L) (Jackson ImmunoResearch; dilution 1:20) for 45 min and with Cy3-conjugated streptavidin (Jackson ImmunoResearch, dilution 1:1250) for 30 min. Each incubation step was followed by three rinses with PBS. The sections were re-blocked with MouseFab for 30 min and labeled overnight with eGFP-specific rabbit polyclonal antiserum (IgG fraction; Invitrogen; dilution 1:200) followed by Alexa 488-linked donkey anti-rabbit IgG (H + L) antibodies (Invitrogen; dilution 1:200) for 1 h. Next, the sections were stained for 10 min at room temperature with 1 μg/mL of Hoechst 33342 (Invitrogen) in PBS, washed thrice with PBS to remove excess dye and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Light microscopic analysis was performed with a Leica DM5500 B fluorescence microscope (Leica Microsystems, Rijswijk, the Netherlands). Images were captured with a CoolSNAP K4 CCD camera (Photometrics, Tuscon, AZ) and archived using home-made software.

Statistical analysis

Results are expressed as mean ± SD. The level of statistical significance was determined by one- or two-way analysis of variance (ANOVA) followed by Bonferroni's *t* test for multiple comparisons, using Prism software (GraphPad Software Inc., San Diego, CA). A P value < 0.05 was considered significant.

RESULTS

Regeneration of ectopically implanted fresh human and mouse skeletal muscle tissue

In an initial experiment designed to set up the model, the regeneration kinetics of fresh human and BALB/c mouse muscle mince implanted under the skin of NOD/SCID mice were compared starting at day 7 after grafting when the implant is well-vascularized[14,15].

The histological images of multiple tissue sections of the excised implants stained with HPS were used to visualize the morphology and composition of the implants.

In all cases the implants were encapsulated by dense connective tissue as identified by the light orange saffron stain (Figure 1A and 1B1). The central part of the implants consisted generally of anuclear myofibers (Figure 1C11 and 15) a characteristic of myofiber degeneration. In sections labeled with the desmin-specific antibody, areas of skeletal muscle regeneration[16] could be distinguished from non-

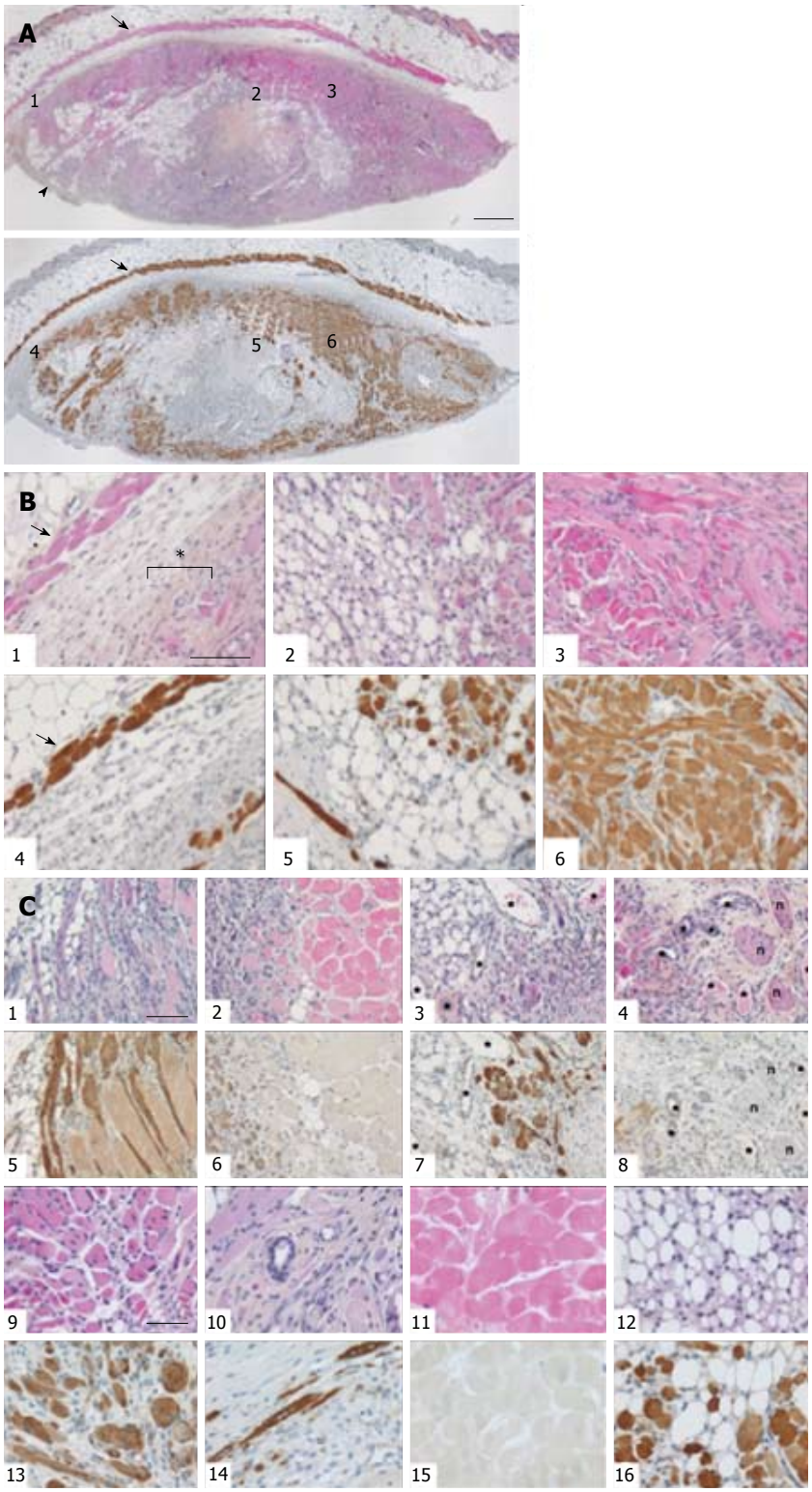

Figure 1 Histological analysis of subcutaneous murine muscle implants. A: Representative images of HPS-stained (upper panel) and desmin-stained (lower panel) sections of a cryopreserved mouse mince muscle implant at 30 d after implantation. From top to bottom are visible the skin layers (epidermis, dermis and hypodermis), the panniculus carnosus muscle (PC, arrow), a thin layer of connective tissue surrounding the implant (light yellow, arrowhead) and tissues composing the implant (*e.g.*, adipose, skeletal muscle, nerves and other tissues). The intense brown desmin stain (lower panel) identifies myofibers of the PC (host) and myoblasts as well as regenerating myofibers of the implant. Scale bar is 500 μm; B: Higher magnifications of the marked areas in A. (1 and 4), connective tissue encapsulating the implant (asterisk). Arrows indicate PC. (2 and 5), adipose tissue and myoblast/myofibers in the central part of the implant. (3 and 6), areas of regeneration with myofibers positioned at different angles. Scale bar is 100 μm; C: Exemplary areas showing specific cells and structures as observed both in fresh and cryopreserved implants. For each pair of images the upper panels correspond to HPS- and desmin-stained tissue sections, respectively. (1, 5, 2 and 6), myoblasts and newly formed myofibers of different sizes and positioned at different angles. (3, 7, 4 and 8), blood vessels (asterisks) and nerves (n) usually located at the edges of the implants. (9, 13, 10 and 14), areas of active regeneration with centronucleated myofibers of different sizes. (11 and 15), degenerated myofibers devoid of nuclei and desmin. (12 and 16), myoregeneration within an adipogenic area. Scale bar is 100 μm for 1-8 and 50 μm for 9-16 is 50 μm. Note the images of HPS- and desmin-stained tissue sections do not always show overlapping areas.

regenerating areas in the implants by the intense brown staining of myoblasts and myofibers (Figure 1B4, 5 and 6). Myoregeneration typically started at the periphery of the implants within the well vascularized connective tissue (see below) and extended with time towards the center (Figure 1B3 and 6). In the areas of myoregeneration satellite cells (Figure 2A), myoblasts and multinucleated myofibers of different sizes and irregularly distributed were observed (Figure 1B3 and 6, 1C1 and 4).

To investigate the vascularization of the implants, sections were immunostained for the endothelial cell marker vWF. Small blood vessels and capillaries staining positive for the endothelial cell marker vWF (Figure 3) were identified as early as at day 7 only in the periphery of the implants but later also in the inner parts. All implants also contained adipose and fibrotic tissues albeit in different amounts and at a different

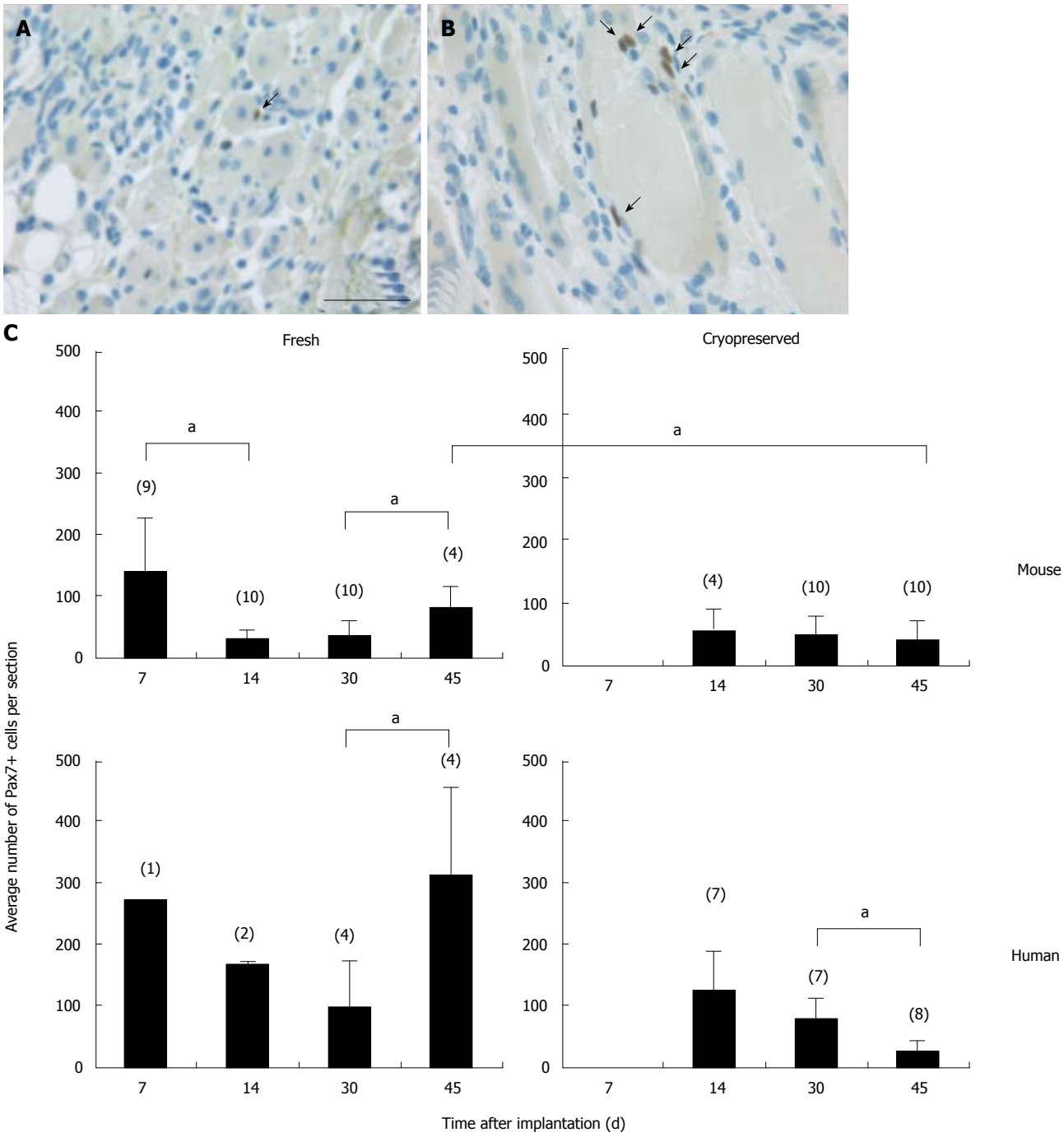

Figure 2 Pax7$^+$ cells in regenerating skeletal muscle implants. A, B: Examples of single (A and B) and pairs of (B) Pax7$^+$ cells (arrows) attached or positioned in close proximity to myofibers in a fresh mouse implant at 14 d after implantation. Scale bar is 50 μm; C: Pax7$^+$ cell counts in cross sections of fresh and cryopreserved mouse and human implants. From the center of each implant two or three consecutive sections were stained for Pax7. The average number of Pax7$^+$ cells per section and SD are plotted. Data from implants with or without MSCs were pooled as they contained very similar numbers of Pax7$^+$ cells. Numbers in parentheses indicate numbers of implants analyzed. $^a P < 0.05$.

distribution (Figures 1 and 4). In general, large differences in the quantities of adipose tissue, fibrotic tissue and skeletal muscle tissue (degenerating and regenerating) were observed even between implants derived from the same donor.

A chronological study of myoregeneration revealed differences in the progression of this process between human and murine implants (Figure 5).

In the fresh mouse muscle implants, shortly after transplantation (day 7), the thin peripheral rim of regenerated tissue consisted predominantly of mononucleated myoblasts. Progression of the regenerative process was evident by the occurrence of elongated multinucleated myofibers at days 14 and 30 post transplantation (Figure 4). The regenerating area gradually extended to occupy 50% to 70% of the implants (Figure 5) Pax7$^+$ cells were detected in all implants, mostly in the periphery. Their number

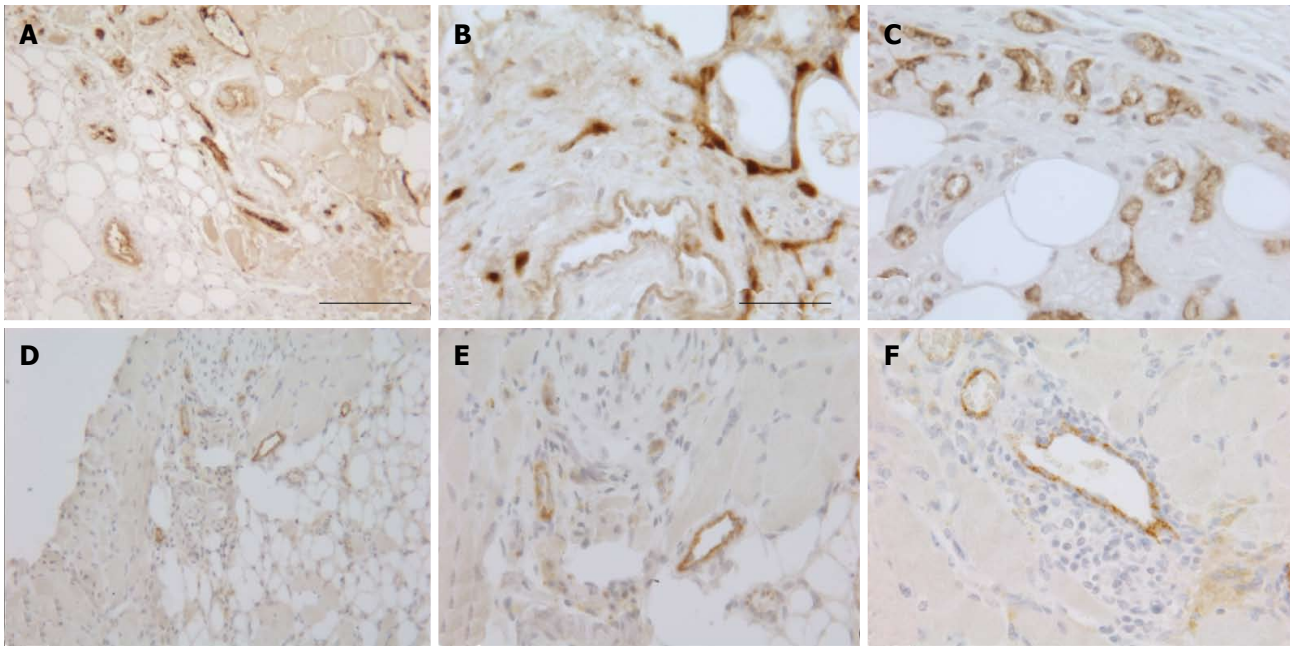

Figure 3 Vascularization of subcutaneous skeletal muscle implants. Photomicrographs of sections of fresh human (A-C) and mouse (D-F) minced implants at 30 d after transplantation stained for vWF (brown). Scale bar is 100 μm for (A, D) and 50 μm for (B, C, E, F).

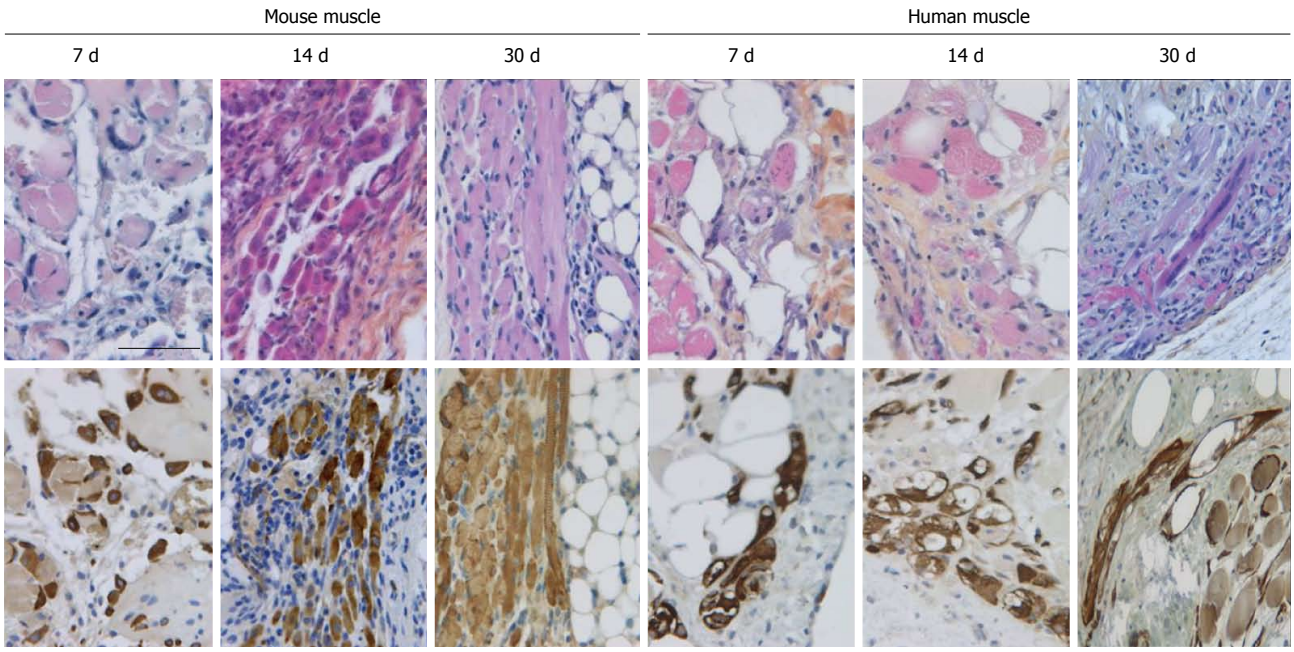

Figure 4 Myoregeneration of mouse and human minced cryopreserved muscle implants. Mouse and human fresh muscle implants were excised at different time points and stained with HPS (upper panels) or for desmin (lower panels). In the mouse implants progression of myoregeneration over time from myoblasts to small and large myofibers was evident. In the human implants, fewer myoblast/myofibers were identified at all time points. Scale bar is 50 μm.

in murine donor tissue was highest at day 7 after implantation (Figure 2C). The observed decrease in the number of $Pax7^+$ cells at later time points (day 30) coincided with an increase in myoregeneration in the fresh murine muscle implants (compare Figure 2C with Figure 5).

The regeneration process in the fresh human muscle implants resembled that in the murine implants, albeit at a slower rate (Figures 4 and 5). The extent of regeneration as deduced from the number of myoblasts and regenerated myofibers in the human tissue at day 30 and 45 after implantation was lower than in the murine muscle at day 14 post transplantation. Notably, the number of satellite (*i.e.*, $Pax7^+$) cells in the human muscle implants was twice to four times as high as that observed in the murine muscle tissue (Figure 2C).

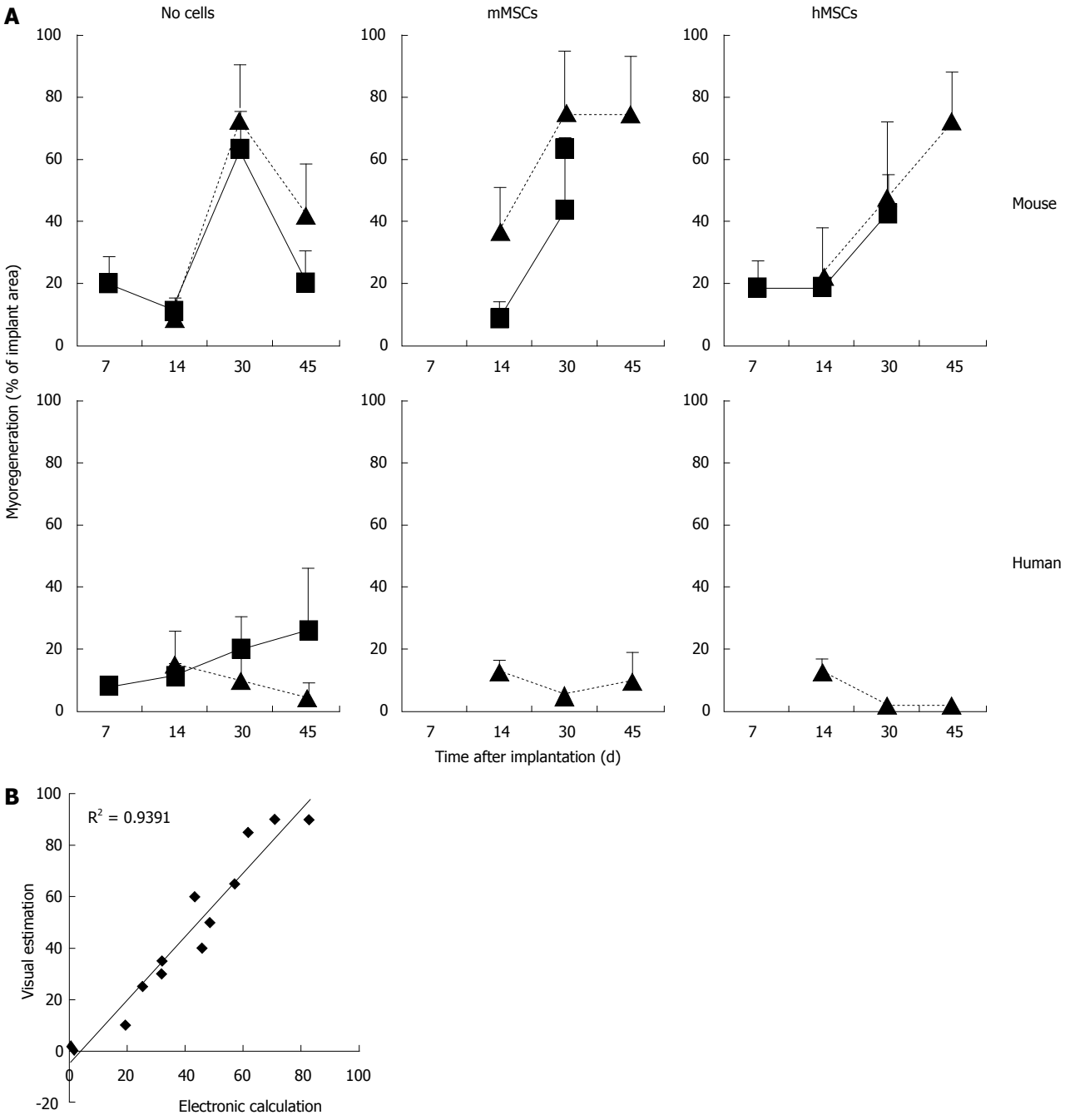

Figure 5 Myoregeneration kinetics of minced muscle implants without and with mesenchymal stem cells. A: The data represent visual estimates derived from microscopic examination of 2 or 3 desmin-stained sections through the center of fresh (square frame and solid line) and cryopreserved (triangle and broken line) implants. Each point represents the mean with SD of between 2 and 10 (average 4) implants; B: Comparison between visual estimation and electronic calculation of areas of myoregeneration. Thirteen desmin-stained cross sections were analyzed by both visual estimation (plotted on the Y-axis) and with the Interactive Image Measurement program of CELL^F imaging software (plotted on the X-axis). The excellent correlation between the two methods of analysis justifies the choice of the less laborious visual estimation procedure.

Regeneration of cryopreserved muscle tissue

Because of the logistic problems caused by the irregular supply of human muscle samples and their limited size, we explored the option of using cryopreserved tissue. This approach allows also pooling of samples collected at different dates and from different donors, a strategy that might improve reproducibility.

The regeneration of cryopreserved mouse muscle closely resembled that of the fresh implants as judged by HPS and desmin stainings (Figure 4). The frequency of $Pax7^+$ cells (Figure 2C) was not much affected by the freezing procedure, except for day 45 ($P < 0.05$). Human muscle cryopreserved in an identical manner behaved differently. At 14 d after transplantation, the ingrowth of capillaries into the implant and signs of myoregeneration were observed at the edges of the donor tissue like in implants of fresh tissue. At later time points (days 30 and 45),

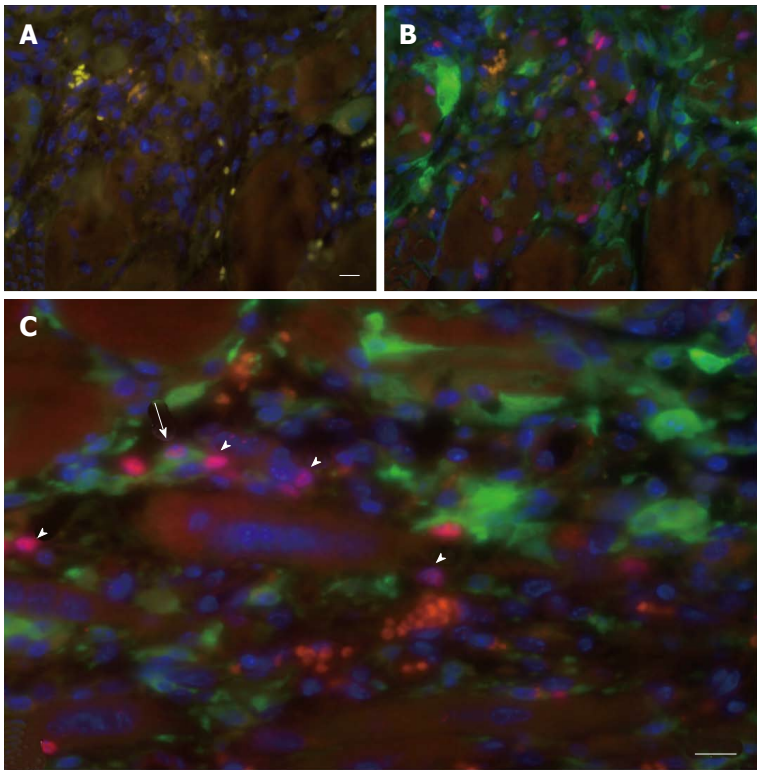

Figure 6 Contribution of host cells to myoregeneration in the muscle implant. Immunofluorescence analysis of a fresh mouse implant excised 7 d after implantation in eGFP transgenic recipients. Host cell contribution was evaluated in tissue sections stained with the karyophilic fluorochrome Hoechst 33342 and antibodies specific for eGFP and Pax7. A: Negative control section incubated with Hoechst 33342 (blue), the secondary antibodies and Cy3-conjugated streptavidin; B: Section stained for Pax7 (red), eGFP (green) and Hoechst 33342 (blue); C: Example of a cell positive for both Pax7 and eGFP (arrow) situated at the periphery of the implant. Also present are cells only positive for Pax7 (arrowheads) or for GFP, blood vessels with erythrocytes (orange) and multinucleated myofibers (center of image). Scale bar for a and b is 50 μm. Panel (C) shows an electronic enlargement of an image recorded at the same magnification as (A) and (B).

however, the implants from frozen tissue consisted mostly of fibrotic and/or adipose tissue. Degenerating muscle tissue gradually disappeared while the level of newly formed myoblasts/myofibers remained low (Figure 4). In contrast to the fresh muscle implants, the number of satellite cells decreased with time (Figure 2B), suggesting damage to the satellite cell population by the preservation procedure.

Contribution of host cells to myoregeneration in subcutaneous muscle implants

The skin of a mouse, like that of most rodents, contains a thin skeletal muscle layer named the panniculus carnosus. As some damage of the panniculus carnosus during the implantation procedure is unavoidable, a possible contamination of the graft with recipient satellite cells had to be taken into consideration. This was investigated using eGFP-expressing transgenic hosts that were implanted with skeletal muscle tissue of syngeneic C57BL/6 mice. The minced muscle implants were analyzed 7 d later for the presence of eGFP+ cells expressing Pax7, indicative for contamination of the implant with host-derived satellite cell. A large number of eGFP+ mononucleated cells was present in the implants but only a few isolated cells co-expressed Pax7 (Figure 6). These findings demonstrate a negligible contribution of recipient cells to the myoregenerative process in the implants and are in agreement with previous reports[7,17].

The evident profusion of erythrocytes and host-derived mononuclear cells in the implants underlines the functional vascularization of the graft already early after transplantation.

Effect of syngeneic or xenogeneic MSCs on myoregeneration in muscle implants

Addition of BM-derived LacZ-tagged MSCs of either species to minced muscle tissue (fresh or cryopreserved) prior to implantation did not consistently affect the degree or the kinetics of myoregeneration to any significant extent (Figure 5A).

The presence of MSCs in the implants and their contribution to the myofiber formation was evaluated using sections stained with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal). β-galactosidase-positive (b-gal+, blue color) cells were present generally in all sections. Their frequency, distribution, and incorporation into multinucleated myofibers were different in the various implants. To enable comparison between the different treated groups we used an arbitrary score ranging from 0 to 4, where (0) no blue cells; (1) single blue cells only; (2) in addition clusters of blue cells; (3) in addition clusters of blue cells and some blue myofibers; and (4) in addition clusters of blue myofibers. The persistence of MSCs and their participation in myofiber formation were similar for the mMSC and the hMSC in cryopreserved murine muscle implants as well as for mMSC in fresh mouse muscle implants. In contrast, hMSCs were strikingly less abundant in implants of fresh mouse muscle and did not contribute to myofiber formation at any time points (Figure 7A). While numerous blue myofibers were recorded in implants supplemented with syngeneic MSCs, only isolated blue cells were detected in the implants supplemented with the xenogeneic cells (Figure 7B). The higher contribution of the mMSCs as compared to the hMSCs is in line with findings in the

cardiotoxin (CTX)-damage *in vivo* mouse model of the tibialis anterior muscle[2] where hMSCs were half as effective in producing β-gal+ myofibers as mMSCs (Figure 7C).

The contribution of MSCs to human skeletal muscle regeneration could only be investigated in cryopreserved muscle. Supplementation of the implants with mMSCs or hMSCs did not lead to the formation of β-gal+ myofibers at any of the time points (Figure 7A). Under all conditions, MSCs persisted in the implants as isolated or clustered mononuclear cells (score 2 or lower).

Myofiber calcification and cartilage formation in subcutaneous muscle implants

A thorough analysis of the histological sections of the various muscle implants revealed the presence of cellular structures with a deviating morphology. Additional specific staining with Alizarin Red S confirmed calcification of myofibers in 50 of 66 analyzed human muscle implants (Figure 8A1, 2 and B). This calcification, which occurred predominantly in degenerated myofibers, was seen in implants of both fresh and cryopreserved human muscle tissue either supplemented or not with MSCs.

In 10 of 81 of the murine muscle implants we identified isolated islands of encapsulated cartilage (Figure 8A3, 4 and B) by staining with Alcian Blue. This occurred in implants with as well as without MSCs. Calcification has not been recorded in any of the murine implants. Similarly, we have not found chondrogenesis in the human muscle implants at any time point.

DISCUSSION

A major impediment to the development of stem cell therapy for myogenic disorders is the paucity of models for studying regeneration of human skeletal muscle. The minced muscle implants described here have provided information of translational significance: firstly, by confirming *in vivo* observations[18] that regeneration perse is slower for human muscle than for murine muscle, and secondly, by demonstrating that MSCs have little or no effect on the rate of myoregeneration. This observation corroborates previous findings from our research group with human BM-derived MSCs and CTX-injured murine tibialis anterior muscles[2]. Thirdly, by revealing that the contribution of hMSCs to the regeneration of fresh mouse muscle implants is far below that of implants of cryopreserved murine muscle, suggesting that the results obtained with the current *in vivo* models, which are based on the direct injection of hMSCs into damaged mouse muscle, their myoregenerative capacity in humans may be underestimated. Based on this finding it is tempting to speculate that species-specific inhibitors in the mouse tissue that become deactivated by the cryopreservation may be present. This model, may, then, offer excellent opportunities for identifying such inhibitors and evaluating their significance for tissue regeneration.

Furthermore, the contribution of MSCs to myofiber formation seems to occur only in tissues undergoing massive myoregeneration, like the murine implants at 30 and 45 d post transplantation. In tissues showing no or little evidence of regeneration, like the cryopreserved human muscle implants at day 30 after transplantation, β-gal+ myofibers were not detected. The observation that MSCs are maintained in the damaged muscle environment as mononucleated cells without contributing to the active satellite cell pool or forming homotypic myofibers may argue against MSCs having autonomous myogenic differentiation capacity. The β-gal+ myofibers observed in the murine implants result solely from fusion of MSCs with nascent or regenerating implant-derived myofibers.

This conclusion is in line with the notion that the intrinsic differentiation potential of MSCs is limited to certain connective cell types including osteoblasts, chondrocytes, adipocytes, fibroblasts and adventitial reticular cells[19,20]. Evidence provided so far for the ability of MSCs to differentiate along the myogenic lineage is conflicting. Although some previous studies assigned myogenic properties to MSCs by demonstrating their *in vitro* and *in vivo* differentiation into satellite cells and myoblasts and their ability to form myotube-like structures through homotypic fusion[19,21], others regard the myogenic reprogramming of the MSCs to be the consequence of their fusion with inherently myogenic cells[2,22,23]. Whether this contradiction can be attributed to the differences in MSC origin, the model used or the read-out methods applied, remains to be investigated.

Implantation experiments revealed that cryopreservation was detrimental to the recovery of human satellite cells but not to that of the murine cells. Regeneration of cryopreserved murine muscle was the same as that of fresh tissue. These results were rather unexpected. Such difference was not recorded between human and murine cells of other tissues (*e.g.*, hematopoietic stem cells and MSCs). To take this study forward, efforts will have to be made to improve human satellite cell viability and proliferation potential. In the development of the cryopreservation protocols reduction of the concentration of DMSO or even its omission from the preservation mixtures should be considered, according to a recent report[24].

The finding of hyaline cartilage in the murine implants and calcified myofibers in the human muscle implants, was both intriguing and has so far not been described. The myofiber calcification without evident bone-like trabeculae formation, that was observed in 75% of the human muscle implants, may represent dystrophic calcification that often occurs in necrotic soft tissues[25,26]. The rapid onset of myoregeneration in the murine muscle implants and its progression through the entire implant, leaving only few degenerating myofibers, might explain the absence of calcification in the murine implants.

The origin of the cartilage, however, is more difficult

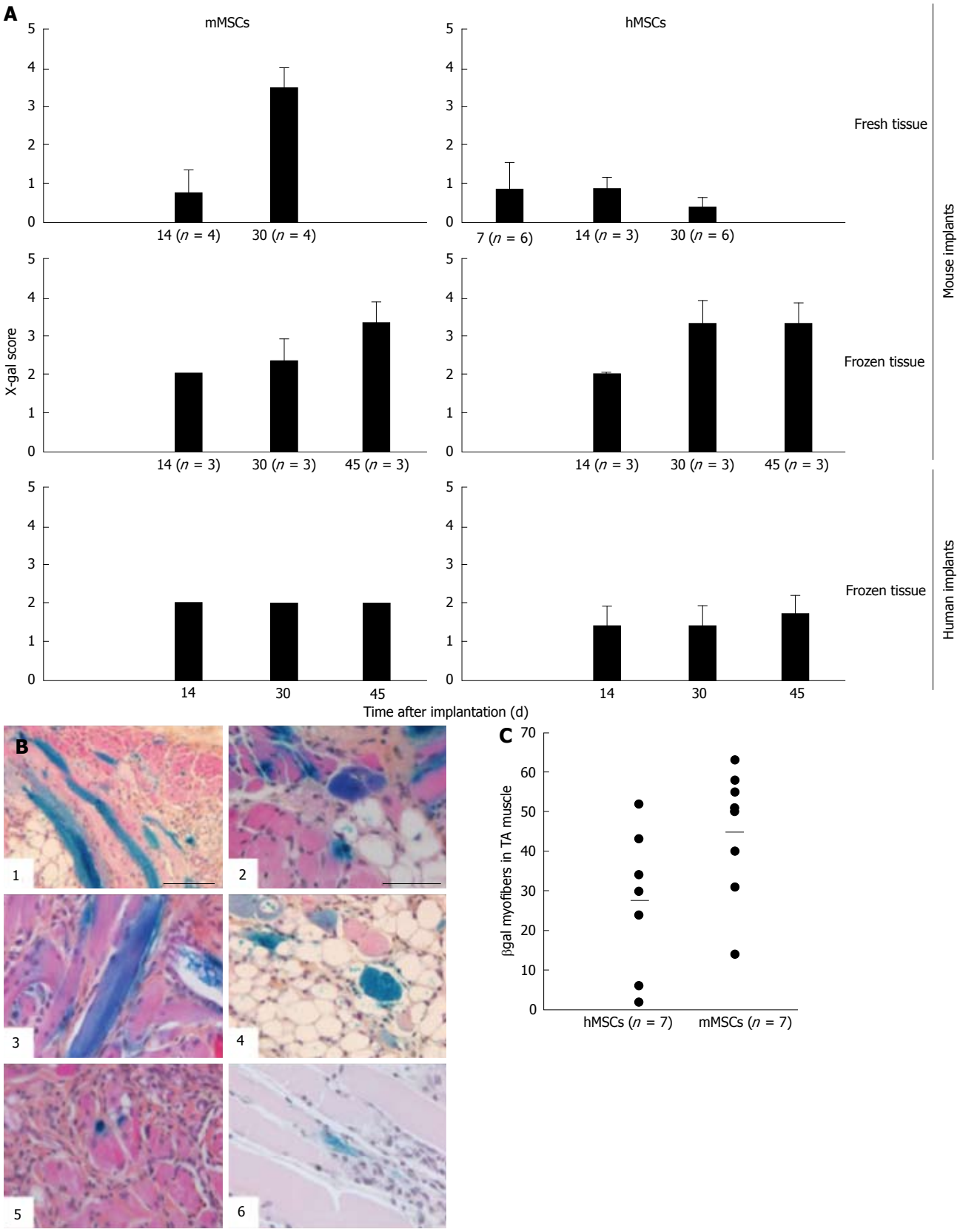

Figure 7 Contribution of mouse mesenchymal stem cells and human mesenchymal stem cells to myoregeneration in implants of fresh and cryopreserved mouse and human muscle minced. MSCs and their derivatives were identified by X-gal staining. A: For comparing implants we used an arbitrary score described in Results. Plotted in A is the average X-gal score and SD. Numbers in parentheses indicate number of implants analyzed; B: Representative images of X-gal-stained sections of fresh mouse muscle implants at 30 d after grafting. Note the higher frequency of blue myofibers (longitudinal and transversal cuts) and β-gal$^+$ mononuclear cells in the implants containing mMSCs (B1-4) as compared to those with hMSCs (B5 and 6). Scale bar is 100 μm for B1 and 50 μm for B2 through B6; C: Quantification of β-gal$^+$ myofibers in CTX-damaged tibialis anterior (TA) muscles of NOD/SCID mice following injection of LacZ-tagged MSCs. Each mouse received 5 × 10^5 cells. Muscles were collected 30 d after stem cell injection and processed as described in[2]. Data points represent numbers of hybrid myofibers per TA. The average number of hybrid myofibers for hMSCs and mMSCs was 27 ± 18.3 and 45 ± 16.2, respectively. MSCs: Mesenchymal stem cells; hMSCs: Human mesenchymal stem cells; mMSCs: Mouse mesenchymal stem cells.

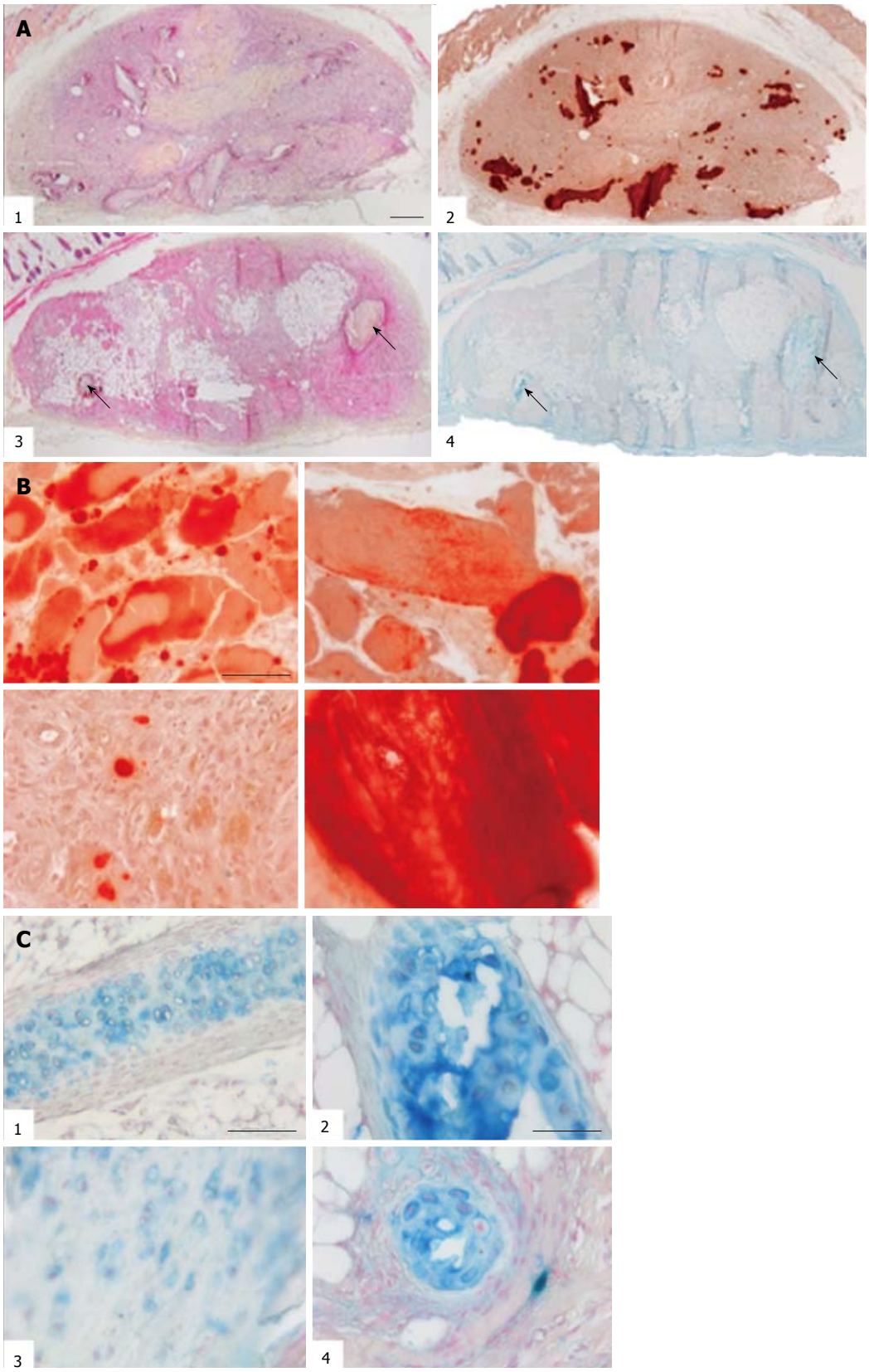

Figure 8 Myofiber calcification and cartilage formation in skeletal muscle implants. A: Human (1, 2) and murine (3, 4) muscle implants at 30 d after grafting stained with HPS (1, 3), Alizarin Red S for bone (2) or Alcian Blue for cartilage (4). Arrows indicate cartilage. Scale bar is 500 μm; B: Larger magnifications of Alizarin Red S-stained myofibers in human muscle implants. Scale bar is 50 μm; C: Larger magnifications of Alcian Blue-stained murine implants. (1), mouse sternum (positive control for the staining procedure), (2-4), examples of chondrogenic tissue in murine muscle grafts. Scale bar is 100 μm for (1) and 50 μm for (2-4).

to explain. It was often seen within the adipose tissue of the implants an environment that, to our knowledge, has not been reported for aberrant cartilage formation before.

In summary, our primary question of whether the contribution of MSCs to myoregeneration is subject to species barriers could be partly answered through the use of ectopically implanted minced muscle. While hMSCs failed to participate in the regeneration of fresh mouse muscle implants, they did contribute to the regeneration of murine skeletal muscle tissue that had been cryopreserved prior to implantation. Collectively, our data indicate that the *in vivo* model described herein is valuable for studying some aspects of human skeletal muscle degeneration and regeneration. A major practical issue to be tackled is the cryopreservation of human skeletal muscle for which the current standard technique was found to fail.

COMMENTS

Background

The translational relevance of animal models for tissue repair is often ambiguous. The authors describe here a murine model for the comparison of the regeneration of damaged human and murine skeletal muscle implants and the contribution of human and mouse mesenchymal stem cells (MSCs) to this process. These cells were mixed with minced muscle prior to subcutaneous implantation in mice, this allows for an equal distribution of the MSCs in the muscle mass. The added value of the present model is that it permits the dissection of species-specific factors in the microenvironment.

Research frontiers

The recent advances in (1) the derivation of human pluripotent stem cells; (2) the characterization and *ex vivo* amplification of human somatic stem cells; and (3) the genetic modification of these cells have created new prospects for cell-based therapies. A major impediment to the development of stem cell therapy for myogenic disorders is the paucity of animal models for studying regeneration of human skeletal muscle.

Innovations and breakthroughs

After transplantation of different human stem cell types including pericytes, satellite cells, MSCs and muscle precursor cells into damaged murine skeletal muscle, typically 1%-7% of the myofibers in the regenerated tissue contained human nuclei. Similar experiments performed with allogeneic satellite cells injected into muscles of mdx mice (a mouse model for Duchenne muscular dystrophy) showed more than 10% chimeric myofibers after the administration of a significantly smaller cell dose than was used for the xenotransplantation studies. The reconstitution frequency by syngeneic donor cells was even more profound in mdx mice transplanted with a subpopulation of satellite cells with 94% of all myofibers becoming chimeric. Although these findings require confirmation by direct comparative studies, they suggest a higher propensity of murine than of human (stem) cells to participate in the regeneration of mouse skeletal muscle tissue. Consequently, the results of preclinical studies with human stem cells in mice may lead to an underestimation of their therapeutic potential in humans. The present study is an attempt to develop a method for investigating this hypothesis.

Applications

These findings suggest that fresh murine muscle tissue provides a suboptimal environment for maintenance of human MSC, and that in *in vivo* mouse models their capacity to engage in myoregeneration is underestimated. The added value of the present model is that it permits the dissection of species-specific factors in the microenvironment. The broader application of this model requires the development of improved methods to cryopreserve satellite cells in human skeletal muscle.

Terminology

The study includes both human and murine muscle grafts, both fresh and cryopreseved, supplemented with either mouse- or human bone marrow-derived MSCs. Non-obese diabetic/severe combined immunodeficient mice served as hosts. Implantation of minced muscle was subcutaneous (under the skin) that facilitates tracking of implant and its removal.

Peer-review

The study conducted by de la Garza-Rodea *et al* described a murine model using subcutaneous implants of minced muscle for examining the regeneration of damaged human and murine skeletal muscle implants and the contribution of added corresponding human and mouse mesenchymal stem cells. The authors concluded that (1) the contribution of human mesenchymal stem cells to murine myofiber formation was restricted to the cryopreserved mouse muscle implants suggesting that fresh murine muscle tissue provided a suboptimal environment for the maintenance of human mesenchymal stem cells; and (2) their described model allowed the dissection of species-specific factors in the microenvironment. The authors commented that the application of their described model requires the development of improved methods to cryopreserve satellite cells in human skeletal muscle.

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EDITORIAL

Interferon-γ: Promising therapeutic target in atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disorder of the vasculature and is the primary cause of cardiovascular disease (CVD). CVD is currently the world's leading cause of death and the numbers are predicted to rise further because of a global increase in risk factors such as diabetes and obesity. Current therapies such as statins have had a major impact in reducing mortality from CVD. However, there is a marked residual CVD risk in patients on statin therapy. It is therefore important to understand the molecular basis of this disease in detail and to develop alternative novel therapeutics. Interferon-γ (IFN-γ) is a pro-inflammatory cytokine that is often regarded as a master regulator of atherosclerosis development. IFN-γ is able to influence several key steps during atherosclerosis development, including pro-inflammatory gene expression, the recruitment of monocytes from the blood to the activated arterial endothelium and plaque stability. This central role of IFN-γ makes it a promising therapeutic target. The purpose of this editorial is to describe the key role IFN-γ plays during atherosclerosis development, as well as discuss potential strategies to target it therapeutically.

Key words: Atherosclerosis; Interferon-γ; Inflammation; Neutralization; MicroRNA

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Core tip: Atherosclerosis is an inflammatory disorder of the vasculature and studies in mouse model systems have highlighted the beneficial effects of counteracting inflammation in limiting the progression of this disease. Due to its key role in inflammation and atherosclerosis development, interferon-γ (IFN-γ) is seen as a promising therapeutic target. In this editorial we discuss the role of IFN-γ in atherosclerosis together with potential therapeutic approaches against this cytokine and its key downstream targets.

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INTRODUCTION

Atherosclerosis is the underlying cause of cardiovascular disease (CVD) such as myocardial infarction (MI) and

stroke. The World Health Organisation estimated that there were 17.5 million deaths from a CVD-related event in 2012, equating to approximately 1 in 3 global deaths[1]. The number of global deaths related to CVD has been predicted to increase due to rises in the incidences of obesity and diabetes and the acquisition of a westernised diet in developing countries. The disease is a major healthcare and economic burden and therefore there is a need to understand the disease in more detail and to develop new therapeutic approaches.

ATHEROSCLEROSIS DEVELOPMENT

Atherosclerosis is a chronic, inflammatory disease characterized by the formation of foam cells in initial atherosclerotic lesions which then progress into advanced plaques. Low-density lipoprotein (LDL) can become trapped in the intima of medium and large arteries and modified to oxidized LDL (OxLDL). The presence of OxLDL triggers an inflammatory response in the neighbouring endothelial cells (ECs), causing the release of a variety of pro-inflammatory cytokines and chemokines, and expression of adhesion molecules on the cell surface (activation of ECs). These factors include macrophage chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) as well as P- and E-selectins[2,3]. Such pro-inflammatory molecules guide circulating monocytes in the blood stream to the OxLDL accumulation in the intima of arterial walls and aid the progression of atherosclerosis development[4-6]. Once in the intima the monocytes become exposed to macrophage colony-stimulating factor, triggering their differentiation into macrophages as well as inducing scavenger receptor (SR) expression on their surface[2,7]. Macrophages are then able to uptake OxLDL by SR-mediated endocytosis, macropinocytosis or phagocytosis and develop into foam cells, causing the appearance of the initial lesions and fatty streaks in arteries, which can then progress into mature plaques[8,9].

Mature atherosclerotic plaques are made up of vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM), as well as accumulated OxLDL, cholesterol and apoptotic cells, which form a lipid-rich necrotic core[10]. During plaque progression VSMCs proliferate and migrate towards the LDL accumulation and form a fibrous cap, which is tightly controlled and influenced by the nearby macrophages, ECs and T-cells[2,11]. As the fibrous cap continues to develop it forms a stable lesion by covering the large lipid-rich necrotic core, therefore the balance of ECM production and degradation can affect the stability of the lesion[2]. If the plaque ruptures it triggers a thrombotic reaction and in turn platelet aggregation, which can quickly impede or obstruct blood flow through the artery[7]. Depending on the location of the rupture it can potentially cause a MI or stroke. Therefore acute CVD events may be manageable by affecting plaque stability and preventing them from rupturing[7,12]. Amongst the cytokines involved in the development of atherosclerosis, interferon-γ (IFN-γ) is potentially a master regulator and will therefore be addressed in more detail.

IFN-γ

IFN-γ is a key pro-inflammatory cytokine in atherosclerosis development as it is capable of inducing the expression of approximately a quarter of genes expressed in macrophages[3]. Immune cells present in the atherosclerotic lesions, including T-lymphocytes, natural killer T-cells, macrophages and other antigen presenting cells, secrete IFN-γ at pronounced levels[13,14]. Stimulation of many signaling pathways that regulate the immune and inflammatory responses can be induced by IFN-γ. The major signaling pathway that IFN-γ signals through is the Janus kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathway[3].

JAK-STAT pathway

The IFN-γ cell surface receptor complex (IFN-γR) is made up of two subunit pairs (IFN-γR1:IFN-γR) which dimerize upon binding of the cytokine[13]. Bound to each subunit are two JAKs 1 and 2, which become activated by phosphorylation of tyrosine residues in the N-terminus in a mainly JAK2-dependent process[15]. Once activated, the JAKs phosphorylate the tails of the IFN-γR which triggers the recruitment of STAT1 monomers from the cytoplasm that then interact with the receptor *via* their src-homology 2 domains[16]. The recruited STAT1 monomers are then phosphorylated by the JAKs at tyrosine 701 and dissociate from the receptor complex to form STAT1:STAT1 homodimers[3]. The dimer is then able to translocate into the nucleus and stimulate the transcription of IFN-γ target genes, such as MCP-1 and ICAM-1, by binding to γ-activated sequence (GAS) elements in their promoters[13,15]. Furthermore, extracellular signal-regulated kinase (ERK) and other kinases are capable of phosphorylating the homodimer at serine 727 for maximal activity[17].

ROLE OF IFN-γ IN ATHEROSCLEROSIS DEVELOPMENT

Therapeutically targeting IFN-γ in order to reduce the incidence of CVD represents a promising avenue due to its pro-inflammatory functions during atherosclerotic plaque formation, including the recruitment of immune cells to the site of OxLDL accumulation, foam cell formation, and plaque development and stability. A 2-fold increase in the size of atherosclerotic lesions has been reported in the Apolipoprotein E (ApoE) deficient mouse model that was injected with recombinant IFN-γ every day, even with a 15% reduction in plasma cholesterol levels[18]. Furthermore, ApoE deficient mice which also lacked IFN-γR showed a reduction in atherosclerosis development, as well as a 60% decrease in lipid

build up in the lesions when fed on a western diet[19]. Deficiency of STAT1 in mouse model systems is also associated with reduced atherosclerosis development and foam cell formation, highlighting the key role of the JAK-STAT1 pathway in IFN-γ signaling during plaque progression[20,21].

Recruitment of immune cells

IFN-γ is a key recruiter of immune cells in the development of atherosclerosis and therefore important in the growth of lesions[22]. IFN-γ has been shown to be localized in atherosclerotic lesions and mice models lacking either IFN-γ or its receptor have been reported to have a reduced cellular content in their lesions[19,23,24]. The expression of key pro-atherogenic chemokines and their receptors, such as MCP-1 that has been detected in atherosclerotic lesions by immunohistochemistry and *in situ* hybridization, can be induced by IFN-γ[25,26]. Mouse models which were deficient for either MCP-1 or its receptor showed a reduced cellular content in lesions, as well as a reduction in the size of the lesions without changes in circulating lipid or lipoprotein levels[25]. IFN-γ can also influence the recruitment of immune cells by inducing the expression of adhesion molecules, such as ICAM-1 and VCAM-1, in ECs during the early stages of atherosclerosis development[27,28].

Foam cell formation

Cholesterol uptake and efflux is carefully balanced during homeostasis of this sterol in healthy cells. The formation of foam cells can be regarded as a pathological imbalance in favour of reduced cholesterol efflux and increased uptake of OxLDL[7,29]. The expression levels of a number of key genes involved in cholesterol metabolism are regulated by IFN-γ, including ApoE, ATP-binding cassette transporter A1 (ABCA1) and acetyl-CoA acetyltransferase 1 (ACAT1)[22]. *In vitro* studies that have incubated macrophage-derived foam cells with IFN-γ have shown a reduction in cholesterol efflux *via* increasing the expression of ACAT1 and attenuating the expression of ABCA1, resulting in increased accumulation of intracellular cholesteryl esters which promote the formation of foam cells[30]. Furthermore, the expression of several key SRs in foam cell development, including SR-A and SR that binds phosphatidylserine and oxidized lipids (SR-SPOX; also known as CXCL16), have been shown to be increased in human THP-1 and primary macrophages stimulated with IFN-γ, resulting in an increased uptake of OxLDL[31-33]. Therefore IFN-γ is capable of altering cholesterol homeostasis towards lower cholesterol efflux and higher retention of OxLDL in macrophages and contributes to foam cell formation.

Plaque progression and stability

IFN-γ can influence a variety of processes involved in the development of the early atherosclerotic lesions into mature plaques as well as their stability. Part of plaque development involves the migration of VSMCs and the formation of the fibrous cap. IFN-γ induces the expression of integrins on the surface of VSMCs which are capable of binding to fibronectin in ECM, triggering the VSMCs to differentiate from their inactive to their proliferative phenotype allowing migration towards the lesion to form the fibrous cap[34]. The stability of atherosclerotic plaques relies on the balance of ECM production and degradation which can also be affected by IFN-γ[2,22]. Foam cell apoptosis is also promoted by IFN-γ and causes them to expel their contents into the intima, contributing to the lipid-rich necrotic core and ECM degradation[35,36]. The balance can be tipped further towards ECM degradation by IFN-γ-mediated inhibition of the expression of several collagen genes, thereby suppressing matrix synthesis by VSMCs and resulting in reduced plaque stability and increased risk of a rupture[7]. ECM degradation can also be triggered by matrix metalloproteinases (MMPs) which are found in atherosclerotic plaques and are often localized to the shoulder regions where a rupture is more likely to occur[37]. MMPs are released by macrophages and VSMCs and their expression can be induced by IFN-γ stimulation[38].

THERAPEUTICALLY TARGETING IFN-γ

Due to the high prevalence of CVD there are a variety of therapeutics designed to reduce various aspects of atherosclerosis development, including decreasing serum cholesterol levels and altering the expression of genes that are involved in cholesterol metabolism or the inflammatory response[3,39]. Statins, the most widely used and successful cholesterol lowering therapy class of drugs, are primarily designed to inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG CoA reductase)[3]. HMG CoA reductase catalyses the rate limiting step in cholesterol biosynthesis, thereby lowering the levels of circulating LDL[40]. However there is a marked residual risk of CVD in patients on statin therapy, with a significant proportion unable to attain their target LDL levels even when receiving the highest recommended dosage, stressing the importance of developing new therapeutics[2,41].

One new potential therapeutic target is IFN-γ due to its key roles in atherosclerosis development. There are currently two strategies that have been developed that either target IFN-γ directly (IFN-γ neutralization) or inhibit its signaling pathways. Statins and agonists of nuclear receptors also attenuate IFN-γ actions in part by modulating its signal transduction pathways[42-44]. In human macrophages, IFN-γ-induced phosphorylation of STAT1 on serine 727 can be blocked using adenosine[45]. Work by Lee *et al*[46] has shown that stimulation of the adenosine A3 receptor with a novel agonist, thio-CL-IB-MECA, resulted in attenuated IFN-γ-induced STAT1-dependent gene expression. Furthermore a naturally occurring phenol in plant extract, resveratrol, is capable of preventing STAT1 phosphorylation at tyrosine 701

or serine 727 as well as JAK2 activation in human macrophages *in vitro*[47]. These compounds represent promising avenues for therapies targeted at the downstream signaling events in the JAK-STAT pathway in order to reduce the pro-inflammatory effects of IFN-γ. Other therapies target IFN-γ *via* alternative signaling pathways, for example, ACS14 (a hydrogen sulphide releasing aspirin) is capable of attenuating the expression of IFN-γ-stimulated CX3 chemokine receptor 1 (CX3CR1) *via* a peroxisome proliferator-activated receptor-γ-dependent mechanism[48]. Hydrogen sulphide has previously been shown to exert anti-atherogenic effects and its use in ACS14 has been shown to reduce atherosclerosis development in ApoE mice models[48,49].

IFN-γ neutralization involves the use of a soluble IFN-γR (sIFN-γR) which acts as decoy receptor to prevent the activation of IFN-γR and in turn the phosphorylation of STAT1 in the JAK-STAT pathway, in effect "neutralizing" the IFN-γ. The approach was first developed by Koga *et al*[50], and demonstrated in ApoE mice which were fed a high fat diet for 8 wk and given two intramuscular injections of a plasmid encoding sIFN-γR at weeks 4 and 6. Compared to the control mice, those that received the sIFN-γR injections had dramatically reduced atherosclerotic lesion size as well as greater plaque stability. This increase in plaque stability was found to be due to an increase in the number of VSMCs in the fibrous cap in addition to greater collagen deposition. Additionally, there was also a decrease in the amount of lipid accumulation and number of macrophages in the necrotic core, which further improved plaque stability and reduced the risk of rupture. Furthermore, neutralizing antibodies have been used for other cytokines such as IL-1β and show great therapeutic promise[51,52], therefore similar strategies could potentially be developed to use antibodies to achieve IFN-γ neutralization.

Although targeting IFN-γ in atherosclerosis development may result in reduced lesion size and improved plaque stability, there are potential drawbacks that need to be assessed before IFN-γ targeting can be recommended therapeutically. The major concern involves the systemic inhibition of IFN-γ due to the major role it performs in the immune response[53]. Sustained universal inhibition of IFN-γ may increase an individual's risk of acquiring intracellular infections and tumour development[53]. On the other hand it may benefit those high-risk patients who are unable to achieve target LDL plasma levels using currently available therapeutics. A possible solution to overcome universal inhibition would be to try and develop a drug delivery system, for example using nanoparticles, that would allow IFN-γ-targeted therapeutics to be delivered to a specific location rather than system wide[53,54].

Another possible solution would be to target further downstream targets of the IFN-γ signaling pathways, either alone or in combination with therapies that target IFN-γ directly. IFN-γ is known to induce the expression of several microRNAs (miRNAs) in addition to having its own expression regulated by miRNAs[55]. miRNAs are short non-coding single-stranded RNAs approximately 19-25 nucleotides in length that are evolutionary conserved in eukaryotic organisms[56]. Evidence is continuously accumulating that indicates that miRNAs are capable of regulating gene expression by inhibiting translation or inducing targeted mRNA degradation[57]. miRNAs have also been found to regulate a number of key steps during atherosclerosis development, including the inflammatory response triggered by IFN-γ[58-60]. One miRNA that is thought to play a key role in atherosclerosis development is miR-155. Evidence for the role of miR-155 in the inflammatory response was found by O'Connell *et al*[61]. miR-155 was the only miRNA out of 200 tested that was considerably up-regulated in primary murine macrophages after being treated with pro-inflammatory stimulants. Additional evidence for the involvement of miR-155 in the inflammatory response comes from studies which have shown its levels to be up-regulated in macrophages in atherosclerotic lesions as well as having an association with increased pro-inflammatory cytokine expression, potentially due to its ability to repress the expression of the Suppressor of Cytokine signaling 1 (*SOCS1*) gene[62-64]. However the specific role miR-155 plays during atherosclerosis is still being debated, with a number of studies reporting miR-155 to exert pro-atherogenic effects in ApoE deficient mouse models[65,66]. Targeting miRNAs, which are either regulated by IFN-γ and are known to be involved in atherosclerosis development or regulate the expression of IFN-γ, may provide an excellent therapeutic avenue that allows specific arterial targeted treatment to reduce atherosclerosis development and improve plaque stability without potential consequences from systemic IFN-γ inhibition.

CONCLUSION

Due to the central role of IFN-γ during atherosclerosis development and plaque stability, along with the expected rise in global rates of CVD-related events, this cytokine represents a promising therapeutic target. Targeting either IFN-γ directly or its signaling pathways in both *in vitro* and *in vivo* studies has shown that directed therapies have the potential of reducing atherosclerosis development. However the potential side effects of long term IFN-γ inhibition still needs to be assessed.

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EDITORIAL

Eurytrematosis: An emerging and neglected disease in South Brazil

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Abstract

The trematodes of the genus *Eurytrema* are low pathogenic pancreatic parasites, but can be related to a decrease in cattle productive performance and eventually death. Parasitized animals develop chronic interstitial pancreatitis and may show a productive performance drop and emaciation. Human infection by *Eurytrema* sp. has already been reported in other countries as an incidental finding during autopsy or routine tests, but the parasite has not been found in humans in Brazil. However, it is possible that a large number of people could be infected, since parasitological tests have low sensitivity and the parasite is neglected as a pathogen for humans and even animals. Attempts to control and treat Eurytrematosis have generally presented low effectiveness. With the aim to control the disease and provide more information regarding its pathogenicity, our research group is developing a number of studies about *Eurytrema* spp. We hope to determine the damage in productivity, as well as, establish an efficient protocol for treatment and control of Eurytrematosis based on immunoprophylaxis and antiparasitical drug therapy.

Key words: Public health; Pathology; Diseases of cattle; Oxidative stress; Parasitical immunoprofilaxis

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Core tip: The trematodes of the genus *Eurytrema* are low pathogenic pancreatic parasites, but can be related to decrease in productive performance and eventually death of infected animals. Human infection has already been reported but could be underestimated in Brazil. With the aim to control the disease and provide more information regarding its pathogenicity, our research group is developing a number of studies about *Eurytrema* spp. We hope to determine the damage in productivity, as well as, establish an efficient protocol for treatment and control of Eurytrematosis based on immunoprophylaxis and antiparasitical drug therapy.

Schwertz CI, Lucca NJ, da Silva AS, Baska P, Bonetto G, Gabriel ME, Centofanti F, Mendes RE. Eurytrematosis: An emerging and neglected disease in South Brazil. *World J Exp Med* 2015; 5(3): 160-163 Available from: URL: http://www.wjgnet.com/2220-315X/full/v5/i3/160.htm DOI: http://dx.doi.org/10.5493/wjem.v5.i3.160

EURYTREMATOSIS: AN EMERGING AND NEGLECTED DISEASE IN SOUTH BRAZIL

The trematodes of the genus *Eurytrema,* belonging to the family Dicrocoeliidae are parasites known as low pathogenic and are generally considered to be an incidental finding at necropsy or at slaughterhouse, but they can be related to a decrease of an infected animals productive performance, and eventually death[1]. Among the species of *Eurytrema* we can highlight *Eurytrema pancreaticum* (*E. pancreaticum*) and *E. coelomaticum,* common parasites of pancreatic ducts of ruminants, occasionally found in the bile ducts and rarely in the small intestine. *E. pancreaticum* has been reported in cattle, goats, sheep, swine, buffaloes, camelids, cervids and human beings, while the *E. coelomaticum* has been reported in cattle, goats, sheep, buffaloes, leporids and camelids[2]. In Brazil the parasites have two different intermediate hosts, the mollusk *Bradybaena similaris* and different species of arthropods from the genus *Conocephalus*[2]. The life cycle is explained in Figure 1.

In areas where the disease occurs, the prevalence among animals is generally high. In a study undertaken by our group[3] we identified a prevalence of 75% of *Eurytrema* sp. in cattle from the West of Santa Catarina State, with 100% of the farms having at least one infected animal. In another Brazilian study, authors found a lower prevalence: between 8.3% and 40.55% in different regions of the State of Paraná (the state north of Santa Catarina) and also observed a seasonal variation in the parasitism[4]. Bassani *et al*[5] found prevalences between 26.9% and 72.9% in the central-west region of Paraná, but in this study a definite seasonal distribution was not observed. Whereas in the State of Rio Grande do Sul (the state south of Santa Catarina) the parasitism by *Eurytrema* sp. is uncommon[6]. In spite of the high prevalence reported in some studies, clinical cases in cattle with emaciation and mortality are sporadic and when occurs affects no more than 3% of the herd[1].

Human infection by *E. pancreaticum* has already been described as an incidental finding at autopsy or in routine coproparasitological tests[7]. There are no published reports in humans in Brazil, but it is possible that a great number of people could be infected, since in general, it is common that routine coproparasitological tests result in false negatives. Similarly to animals, human infection may be subclinical. Another important aspect is that nowadays in medical practice of parasitological diagnosis, many Brazilian physicians request serological tests, instead of the coproparasitological ones, mainly due to the higher sensitivity. However, since *Eurytrema* sp. has not been described as a human pathogen in Brazil, it is not investigated and is likely to be underestimated. Some feeding habits in areas with high prevalence of the parasite seem to be risk factors for human infection. The consumption of wild watercress as salad is common in our region and is an important risk factor for infection by *Fasciola hepatica*[8] and could also be an important risk factor for *Eurytrema* sp.

In Brazil, the pathological pattern of the clinical cases of Eurytrematosis in cattle was described as chronic and progressive weight loss related to interstitial pancreatitis[1,9,10]. In our routine necropsies of cattle that have died for different reasons, in about 92% of the cases *Eurytrema* sp. was found in the pancreatic ducts (Figure 2), most of the times with moderate to severe infections. During histopathology we have observed that the parenchyma of the pancreas is extensively replaced by fibrous connective tissue (Figure 3), which could disrupt the function of the organ.

Attempts to control and treat Eurytrematosis, when applied, generally present low efficiency. This fact seems to be a result of the parasites resistance against the main endoparasiticides used and the high rate of reinfection. Yamamura[11] conducted a trial with four molecules of endoparasiticides commonly used in veterinary practice: nitroxinil, triclabendazole, rafoxanide and closantel, and reported that none of them were efficient. Araújo and Belém[12] reported that albendazole was not efficient against *Eurytrema* sp. in naturally infected cattle, even when administrated in high dosages (17.5 mg/kg bw).

There are few reports about efficient treatments against Eurytrematosis in cattle. Sakamoto *et al*[13] described treatment regimes involving two applications of nitroxinil with an interval of 20 d, and three applications of praziquantel with intervals of two days; both regimes reduced the output of eggs in feces to 0. Jiraungkoorskul *et al*[14] in an *in vitro* trial concluded praziquantel should be the chosen drug for Eurytrematosis control, since it caused the death of 100% of the parasites, while

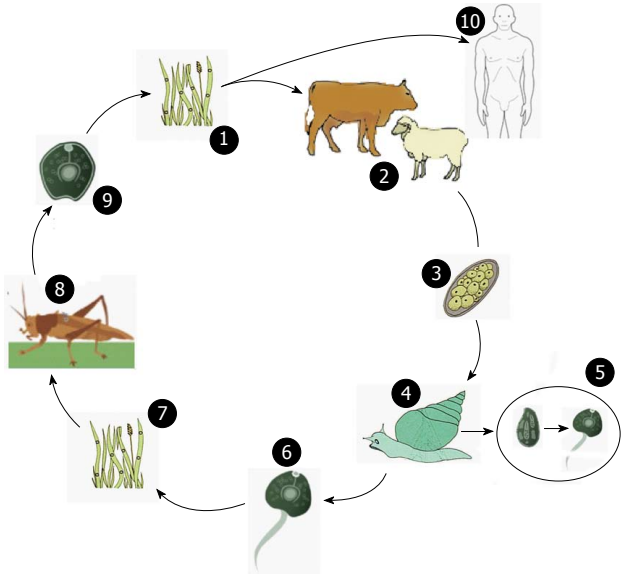

Figure 1 *Eurytrema* spp. life cycle. 1: Infection by grazing on infected grass; 2: Vertebrate host (cow or sheep); 3: Unembryonated eggs released in feces; 4: Ingestion of eggs by *Bradybaena* sp.; 5: Sporocyst mother in sporocyst son in the digestive tube between 90 and 350 d, according to envionmental conditions. Sporocyst sons containing cercarea are released in the environment few hours before dawn; 6: Cercarea released in the environment; 7: Ingestion of cercarea by *Conocephalus* sp.; 8: Development of infective metacercarea inside grasshopper; 9: Infective metacercarea released in the environment; 10: Possible human infection by consumption of infected salad. One egg of *Eurytrema* spp. produces 100 sporocyst sons which turn into 200 cercareas and latter 20000 infective metacercarea. Adapted from Tessele, CDC and Bennett[6,15,16]. CDC: Centers for Disease Control and Prevention.

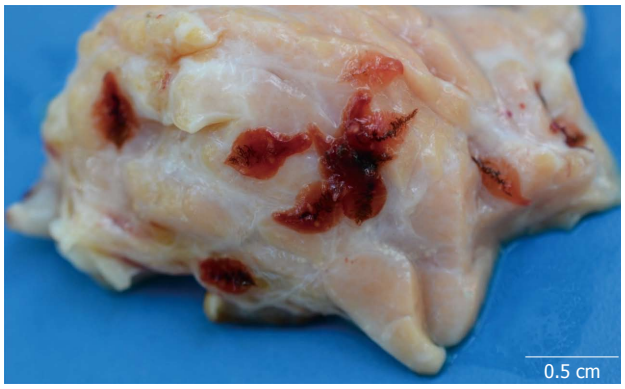

Figure 2 *Eurytrema* sp. in bovine pancreas. The fluke is red, oval shaped and measuring between 8 to 13 mm in length by 6 to 7 mm width. The pancreas of infected animals has a harder consistency and hyperplasic ducts partially filled with adult parasites are observed.

triclabendazole did not present efficacy.

There have been only a few *in vivo* trials aiming to control the disease, and at the moment there are no treatment protocols establishing an appropriate chemotherapy. Even if there are some apparently efficient antiparasitics, the treatment of an infection cannot be accomplished in Brazil. There are no available products containing praziquantel authorized by the regulatory agencies to be administrated in cattle in Brazil, while nitroxinil, according to the current legislation, cannot be applied in dairy cows. Therefore, these two factors make the treatment of Eurytrematosis impossible in dairy cattle in Brazil, as well as in many other countries.

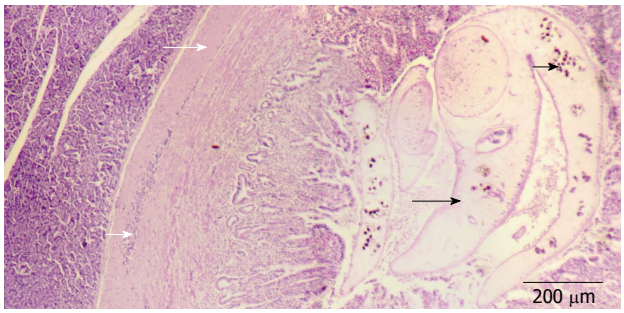

Figure 3 Histopathology of pancreas with Eurytrematosis. Moderate proliferation of connective tissue (white large arrow), severe hyperplasic ducts containing adult parasites (black large arrow), parasite eggs (black short arrow) and mild inflammatory infiltrates of lymphocytes, eosinophils, plasma cells and macrophages (white short arrow). The islets of Langerhans are usually not affected but in most severe cases, they are replaced by connective tissue.

With the aim to control the disease, our group is developing many studies: (1) Molecular determination of the *Eurytrema* species that occurs in cattle and sheep in our region; (2) Establishment of the effectiveness of antiparasitic drugs against *Eurytrema* sp. strains presented in the west of Santa Catarina, Brazil. For this, dairy cattle herds will be segregated into groups, and each group will be treated with a different molecule, taking into account the main drugs used in the region and with known action against trematodes. The second aim of this work is to evaluate if the sub-clinical damage in the pancreatic parenchyma indirectly lead to economic losses; (3) Measure the oxidative stress caused by the parasitism. This comprises gauging the parameters that indicate cellular lesions and the antioxidant status in the pancreas. There are many studies that correlate oxidative stress with clinical-pathologic findings and pathogenesis in infectious diseases. The consequences of infection by *Eurytrema* sp. to an animals' health and productivity have not been elucidated and articles about the oxidative stress in animals parasitized by *Eurytrema* sp. are not available; and (4) Another future study will reproduce the parasites' complete life cycle in the laboratory, aiming to find possible immunogens in the newly encysted juveniles stage of *Eurytrema* sp.

The results of our studies will contribute to elucidate the real importance of Eurytrematosis to animal health and production in Brazil. In the long term, we expect to contribute with directly applicable results to both agribusiness and public health. It is necessary to determine protocols capable of controlling Eurytrematosis in an efficient and viable way. Productivity losses may also be estimated to determine the real economic impact and importance of the disease. We also expect to find different ways to apply a biological and/or immunoprophylaxis control against this parasite, as chemical therapy alone is typically insufficient for the control and prevention of many illnesses, both in

veterinary and human medicine.

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REVIEW

Use of siRNA molecular beacons to detect and attenuate mycobacterial infection in macrophages

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Abstract

Tuberculosis is one of the leading infectious diseases plaguing mankind and is mediated by the facultative pathogen, *Mycobacterium tuberculosis* (MTB). Once the pathogen enters the body, it subverts the host immune defenses and thrives for extended periods of time within the host macrophages in the lung granulomas, a condition called latent tuberculosis (LTB). Persons with LTB are prone to reactivation of the disease when the body's immunity is compromised. Currently there are no reliable and effective diagnosis and treatment options for LTB, which necessitates new research in this area. The mycobacterial proteins and genes mediating the adaptive responses inside the macrophage is largely yet to be determined. Recently, it has been shown that the *mce* operon genes are critical for host cell invasion by the mycobacterium and for establishing a persistent infection in both *in vitro* and in mouse models of tuberculosis. The YrbE and Mce proteins which are encoded by the MTB *mce* operons display high degrees of homology to the permeases and the surface binding protein of the ABC transports, respectively. Similarities in structure and cell surface location impute a role in cell invasion at cholesterol rich regions and immunomodulation. The *mce4* operon is also thought to encode a cholesterol transport system that enables the mycobacterium to derive both energy and carbon from the host membrane lipids and possibly generating virulence mediating metabolites, thus enabling the bacteria in its long term survival within the granuloma. Various deletion mutation studies involving individual or whole *mce* operon genes have shown to be conferring varying degrees of attenuation of infectivity or at times hypervirulence to the host MTB, with the deletion of *mce4A* operon gene conferring the greatest degree of attenuation of virulence. Antisense technology using synthetic siRNAs has been used in knocking down genes in bacteria and over the years this has evolved

into a powerful tool for elucidating the roles of various genes mediating infectivity and survival in mycobacteria. Molecular beacons are a newer class of antisense RNA tagged with a fluorophore/quencher pair and their use for *in vivo* detection and knockdown of mRNA is rapidly gaining popularity.

Key words: Mammalian cell entry; Molecular beacons; siRNA; Mycobacterium tuberculosis; Macrophages

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Core tip: This review paper looks at the current status of research of the role of mammalian cell entry gene products in mediating cholesterol mediated latency of mycobacteria and the potential use of short-interfering RNA molecular beacons in detecting and attenuating mycobacterial infections.

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EPIDEMIOLOGY OF LTB

The year 2005 marked the 100th anniversary since Robert Koch received the Nobel prize for his work on tuberculosis (TB) and yet more than one hundred years later the World Health Organization (WHO) has reaffirmed its designation of TB as a global emergency[1]. Tuberculosis still remains a pandemic, infecting one-third of the world's population and killing millions of people each year. Estimates are that a TB death occurs every minute. According to recent estimates of WHO, nearly 9 million people were infected with TB in 2012, including 1.3 million TB-related deaths worldwide[2]. Incidentally, TB and reactivation of latent TB have turned out to be the leading causes of death for people who are infected with human immunodeficiency virus (HIV). At the same time, according to the Centers for Disease Control (CDC), there were 9945 TB cases reported in the United States in 2012[3]. More than 80% of TB cases in the United States are from reactivation of latent TB infection[4].

Tuberculosis is a disease that spreads from person to person through the air and is mediated by the pathogen *Mycobacterium tuberculosis* (MTB). The TB bacillus was discovered in 1882 and has been the subject of extensive research since then. There is still much to be learned about the nature of this organism, its virulent properties, and its response to host defenses. TB affects the lungs mainly, but can also have other target organs such as brain, spine and the kidneys. When a person with TB infection coughs or sneezes, droplets containing MTB are released into the air and when another person breathes in the infected droplets, they can be infected. However, not everyone infected with TB bacteria becomes sick. There are two TB-related conditions that exist: latent TB (LTB) infection and active TB disease. Those that have latent TB infection do not feel sick or do not present with any symptoms. In LTB cases, even though they are infected with the mycobacterial pathogen, they do not have active TB disease. Overall, it has been shown that about 90% of the people infected with MTB will have LTB infection and 10% will eventually go on to have full-blown active TB at a later stage in their life[5]. Nearly 50% of those who develop TB do so within the first two years of infection. This rate is even higher in immunocompromised individuals, such as those with HIV infection, where the risk of developing TB from LTB activation is significantly higher. Also of particular concern are those infected with drug-resistant TB (XDR TB)[3].

Thus, this ancient human adversary continues to be a challenge in all aspects of medical care, from prevention to diagnosis and therapy.

NEED FOR IMPROVED DIAGNOSTIC AND THERAPEUTIC METHODS FOR LTB

Currently, there are no tests available to directly detect *in vivo* the presence of latent MTB in an affected individual and assessment of latent infection involves an imperfect approach of measuring the host immune response to mycobacterial infection[4]. On the contrary, active TB infection is diagnosed by detecting MTB bacteria in clinical samples taken from patients. A positive diagnosis can be made only by culturing MTB from the specimen, even though the results from this may take four to eight weeks for conclusive answers. Other methods for diagnosing TB include chest X-rays, patient sputum smear microscopy, polymerase chain reaction (PCR) testing, immunological memory-based tests including the less specific purified protein derivative (PPD/tuberculin) skin test and more specific Interferon-γ release assays, phage amplification assays, solid and automated liquid cultures, as well as several tests for antibiotic resistance. These tests can only strongly suggest the presence of active TB or LTB as a diagnosis but they cannot confirm the presence of the bacteria in the body. Reliable and rapid diagnosis of latent TB is a major challenge in low socioeconomic areas, and even in parts of developed countries, especially in areas where immunodeficiency diseases like acquired immune deficiency syndrome are more endemic. In many cases, patients have to undergo time-consuming multiple testing before reaching an apparent diagnosis. This testing deficiency can be especially critical when trying to identify high-risk individuals for prophylactic regimen, and also for identifying and managing extrapulmonary TB sites in HIV co-infected patients[6].

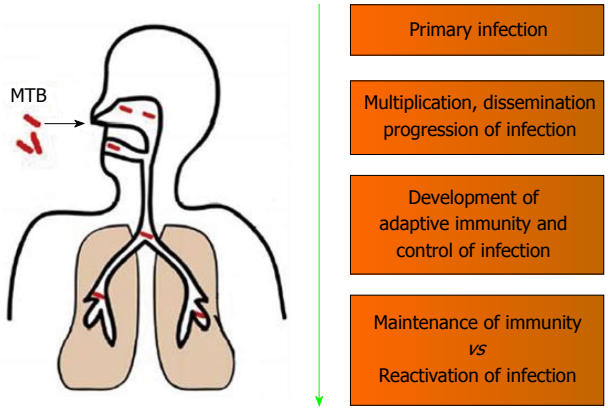

Figure 1 Spectrum of host immune responses against *Mycobacterium tuberculosis*. MTB: *Mycobacterium tuberculosis*.

Treatment for active TB cases consists of a combination of four first-line antibiotics for a period of two months, followed by two drugs for another four months[7]. First line antibiotics consist of rifampicin, isoniazid, pyrazinamide, and ethambutol. These drugs are effective mainly in actively dividing bacilli and its effectiveness in treating LTB, where the bacilli are dormant, has not yet been proven. The treatment of LTB is usually long term with the intent of sterilizing the non-replicative or slowly replicating bacteria[8]. If the particular mycobacterial strain is resistant to the first line drugs, then treatment is escalated for up to 18 mo with five lines of available drugs. Surgery is also performed as a last resort if treatment fails due to drug resistance. Since the chemotherapy regimen for active and latent TB infections usually spans many months, poor patient compliance rates is a major issue contributing to the emergence of resistant strains[4]. There is a pressing need for rapid and inexpensive tests to confirm latent TB cases in order to manage this global epidemic.

Developing a direct MTB imaging screening tool for the asymptomatic population along with novel treatment strategies is vital to our fight against TB. This is especially true for high-risk categories with LTB such as drug users with unsanitary needles, healthcare workers in high risk and densely populated environments, the medically under-served poor and minority populations, children exposed to high risk adults, immunocompromised patients and patients on immunosuppressant drugs, and health care workers who serve these high risk populations[9]. Developing a direct MTB imaging screening tool with combined therapeutic applications for the asymptomatic population is going to have vitally important and far reaching impact in the fight against TB.

SPECTRUM OF HOST IMMUNE RESPONSE AGAINST MTB AND DEVELOPMENT OF LTB

Humans are the only natural host of MTB and are highly susceptible to MTB infections. Even a few (5-10) bacilli is capable of mediating a primary infection[10]. The initial interaction of the MTB with the host involves alveolar macrophages, which is the only known cell type to harbor MTB *in vivo*[11]. Upon coming in contact with MTB, the interaction of the host immune response with MTB can be divided into 4 general types of events[12]: (1) Primary infection event involving the invading MTB; (2) Events that would promote the dissemination and progressions of the MTB infection; (3) Development of an adaptive immunity that would lead to the containment MTB infection; and (4) Interplay of protective immunity involved in latency *vs* immunologic compromises leading to reactivation of MTB infection (Figure 1).

Phagocytosis of the MTB by alveolar macrophages followed by its intracellular growth initiates the cascade of immune events of the primary infection[13]. Activation of the components of the innate immunity, the recruitment of various classes of monocytes and lymphocytes to the site of infection, and the final development of specific immunity allow for the containment of infection (Figure 2).

The hallmark of latent TB is the granulomatous lung parenchymal lesions and their draining lymph nodes which is called the "Ghon complex". The events leading to the formation of granuloma begins when the MTB is inhaled into the lungs and the bacterium is phagocytosed by alveolar macrophages and dendritic cells. The infected cells release proinflammatory cytokines that help recruit more immune cells to the site of infection. The cytokines IL-12 and IL-18 from the infected cells induce natural killer cell activity, which in turn produce IFN-γ that help active macrophages to produce tumor necrosis factor-α and other micobicidal substances. Through the actions of these cytokines and chemokines, other immune cells are recruited leading to the formation of the granuloma[14,15]. In the granuloma, the macrophages further differentiate into epitheloid cells and foamy macrophages and are surrounded by lymphocytes and an outer layer of fibroblasts and matrix proteins. The morphology of the lung granuloma is characterized by a central necrotic core surrounded by concentric layers of macrophages, epitheloid cells, multinucleated Langhans giant cells, and lymphocytes[16,17]. Containment of MTB at the site of primary infection by a cellular wall and a fibrotic outer layer prevents the pathogen from dissemination throughout the host and focuses the immune response to the site of mycobacterial persistence (Figure 3). Successful containment of the pathogen to the site of the primary lesion results in latent infection, which appears in chest X-rays as calcified granulomatous lesions[18].

The exact location of dormant MTB organisms in latent TB has not been elucidated[18]. Studies have shown evidence of the presence of the pathogen in the normal tissue surrounding the granuloma necrotic centers[19] which appears to be the preferred location of the pathogen during latency[20].

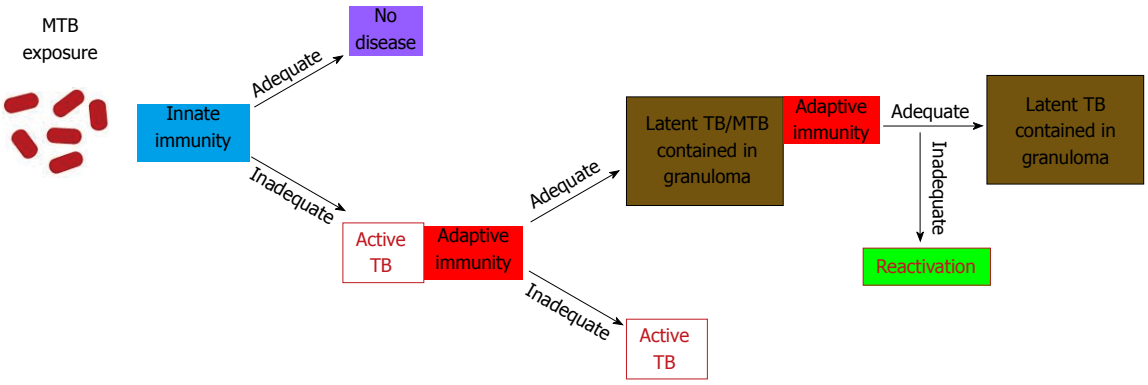

Figure 2 Latent tuberculosis pathogenesis and transmission profile. Most people are adequately protected by innate immunity against MTB infection, however, the infection progresses when the innate immune response levels are inadequate. The development of specific immunity leads to the containment of MTB in granulomas as asymptomatic latent infection. However, inadequate adaptive response at any time progresses to the reactivation and development of full-blown active tuberculosis. MTB: *Mycobacterium tuberculosis*.

A dynamic balance between the host immune response and the MTB pathogen is maintained during latency. Direct cross-talk between MTB and the host immune response occurs in a dense region surrounding the granuloma which is derived from lymphocyte infiltration[21,22]. The granuloma has a central necrotic core which serves as nutritional source for persisting mycobacteria, surrounded by the thick leukocyte wall which prevents the spread of the mycobacteria. The leukocyte derived wall surrounding the granuloma is highly vascularized and facilitates the delivery of drugs against latent TB[23].

POST-PHAGOCYTIC MOLECULAR EVENTS FOLLOWING MYCOBACTERIAL ENTRY

Once entering the host, the ability of MTB to survive decades within the body of the host by subverting the host immune defenses is of continued intrigue and fascination. The precise mechanism of how the bacteria is able to achieve this long term dormancy leading to LTB is still unknown, however, recent advances in mycobacterial molecular biology have shed some light into these processes.

Macrophages make up the major component of the innate host defense, and they do this by pathogen recognition, ingestion and killing of foreign microbes that enters the body including pathogenic and non-pathogenic mycobacteria. The pathogenic mycobacteria have developed a number of strategies to subvert the host immune defenses and evade the destructive action of the macrophages, eventually surviving within this normally inhospitable cell for long periods of time thus resulting in the disease[24]. The surviving bacteria within the macrophages can be in a latent state with stationary growth or, given the right conditions in an immunocompromised host, can switch to a metabolically active state that facilitates proliferation, dissemination and active disease.

Upon gaining entry into the body, most non-pathogenic microbes get phagocytosed by the macrophage into a phagosome where the invading microbe gets exposed to high levels of reactive oxygen species and reactive nitrogen species. The phagosome then goes on to mature and fuse with the organelles of the endocytic pathway, thereby acquiring surface molecular markers which leads to the acidification of the phagosome to pH 5 as well as gaining hydrolytic enzymes that digest the invading microbe[25,26] (Figure 4A). MTB, however, has developed several ways to evade attack by the macrophage and creates a favorable environment for replication (Figure 4B). This is mainly by inhibiting several aspects of phagosomal maturation, including fusion and fission events along the endocytic pathway and the recruitment of vacuolar H^+-ATPases[27,28]. The MTB carrying phagosome retains characteristics of an early phagosome with regard to its pH (about 6-6.5), presence of Rab5 (a Rho-GTPase directing endosomal trafficking and mediating fusion between phagosomes and other organelles), and continued access to other recycling endosomes[29,30], but it lacks mature hydrolases[31] and cathepsins, with interactions between the phagosome and the *trans* Golgi Network blocked[32]. Several MTB products are believed to be inhibitors of phagosomal maturation, including components of the mycobacterial cell envelope such as lipoarabinomannan, trehalose dimycolate and sulpholipids, phosphatase SapM and kinase PknG[29], and the secreted protein ESAT-6[33]. The exact mechanism behind the inhibition of phagosomal maturation by the mycobacteria is yet to be elucidated.

Studies have shown that cholesterol is a necessary component for the uptake of the MTB into the macrophage *via* the complement receptors and for the inhibition of phagosomal maturation[34] (Figure 4B). A host protein associated with the cell membrane called tryptophan-aspartate containing coat protein (TACO) is recruited and retained in the phagosomes harboring mycobacteria thereby preventing the bacterial delivery to lysosomes[35]. TACO is an actin-binding protein seen associated with cholesterol rich regions of the host macrophage plasma membrane[34]. The mycobacterium

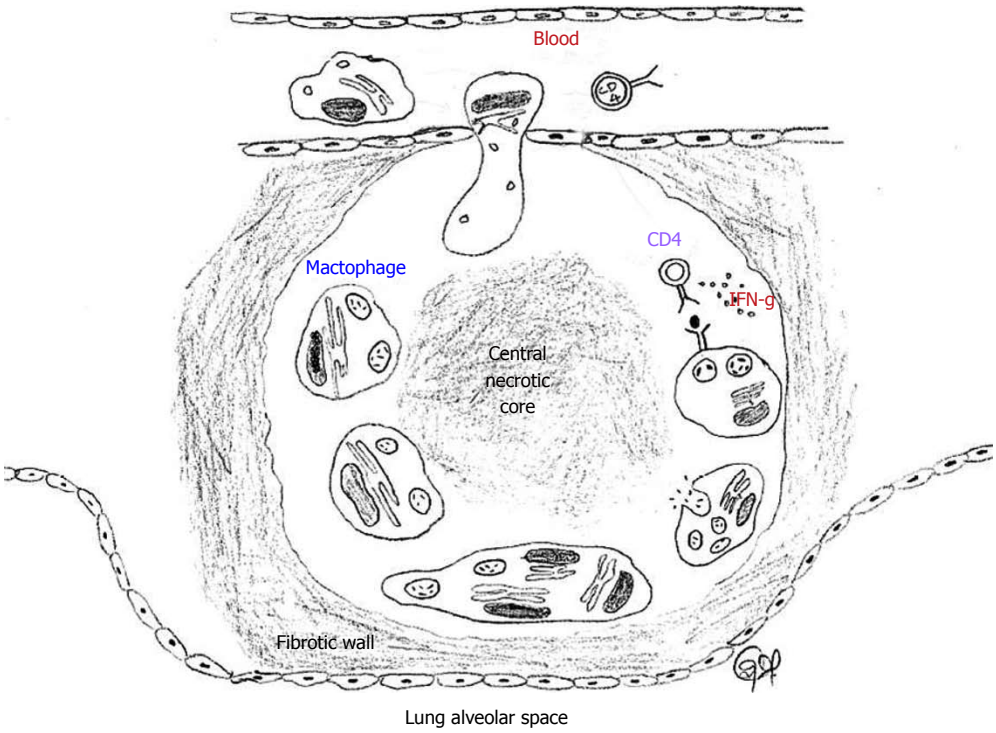

Figure 3 Tuberculosis granuloma. A granuloma sequesters MTB infected macrophages and is surrounded by immune cells, predominantly $CD4^+$ helper T lymphocytes. Some infected macrophages fuse to form foamy giant cells. The infected macrophages and giant cells present antigens to T cells and activate them to produce a variety of cytokines and chemokynes, and also kills the infected macrophage and the MTB. The chemokines also serve to recruit additional T cells to the granuloma from the circulating blood. IFN-γ activates the macrophages to kill the intracellular MTB by generating reactive oxygen species and reactive nitrogen species intracellularly. The center of the granuloma is filled with cell debris and both live and dead MTB spilled from dead macrophages (caseation), all of which form a central hypoxic necrotic core. A sheath of collagen fibers produced from lung fibroblasts surround the granuloma. MTB: *Mycobacterium tuberculosis*.

within the phagosome is somehow able to prevent the removal of the TACO coat protein which prevents the fusion of phagososome with lysosome[35]. Moreover, it has been shown that TACO-mediated uptake of mycobacteria depends on cholesterol[34,36,37]. The mycobacterial proteins and genes mediating these adaptive responses inside the macrophage is largely yet to be determined. MTB′ unique ability to utilize cholesterol, a component of cell membranes, also plays a role in its persistence[38]. In the nutrient-deficient intracellular environment, MTB adapts its metabolism by alternating between carbohydrate and fatty acid metabolism[39]. Studies have shown that MTB utilizes cholesterol for its energy needs and for the biosynthesis of virulence-associated lipid phthiocerol dimycocerosate[38]. A number of reports indicate that MTB metabolizes cholesterol during host infections and the metabolic products contribute to the long-term survival of MTB in the host[38,40,41]. Furthermore, because the cholesterol catabolism pathway requires a large number of oxygenases, it should be no surprise that MTB infects the lungs where oxygen concentration is the highest[42].

MCE OPERONS AND THEIR ROLE IN MYCOBACTERIAL INFECTION AND PERSISTENCE

Over the recent years, it was shown that a DNA fragment from MTB cloned into *Escherichia coli (E. coli)* could mediate the latter's entry and survival in mammalian cells[43] and was named as the mammalian cell entry (*mce*) operon. The *mce* operon genes have been shown to be important in the invasion of the mammalian host cell by the mycobacterium and for establishing a persistent infection both *in vitro* and in mouse models[44,45]. The analysis of the complete genome sequence of MTB in 1998[46] showed that the *mce* operon is composed of a group of four homologous *mce* operons (*mce1, mce2, mce3*, and *mce4*). It was found that all the constituent *mce* genes in the four operons were arranged in an identical manner. Each of the operon contained eight genes, of which two genes preceding the *mce* genes are named *yrbEA* and *yrbEB* which encoded for integral membrane proteins and the six *mce* genes potentially encoding exported proteins (secreted or surface-exposed) thought to be important for the entry and survival of the pathogen in the mammalian cells[46]. The four *mce* operons are widely seen throughout the genus Mycobacterium[34] and the general organization of the genes in each of the four operons are shown in Figure 5.

The YRBE and MCE proteins encoded by the MTB *mce* operons have structural homology to the permeases and the surface binding protein components of the ABC transporters, respectively[47]. The typical ABC permease contains six trans-membrane helices with the C-terminus located on the cytoplasmic side of the membrane

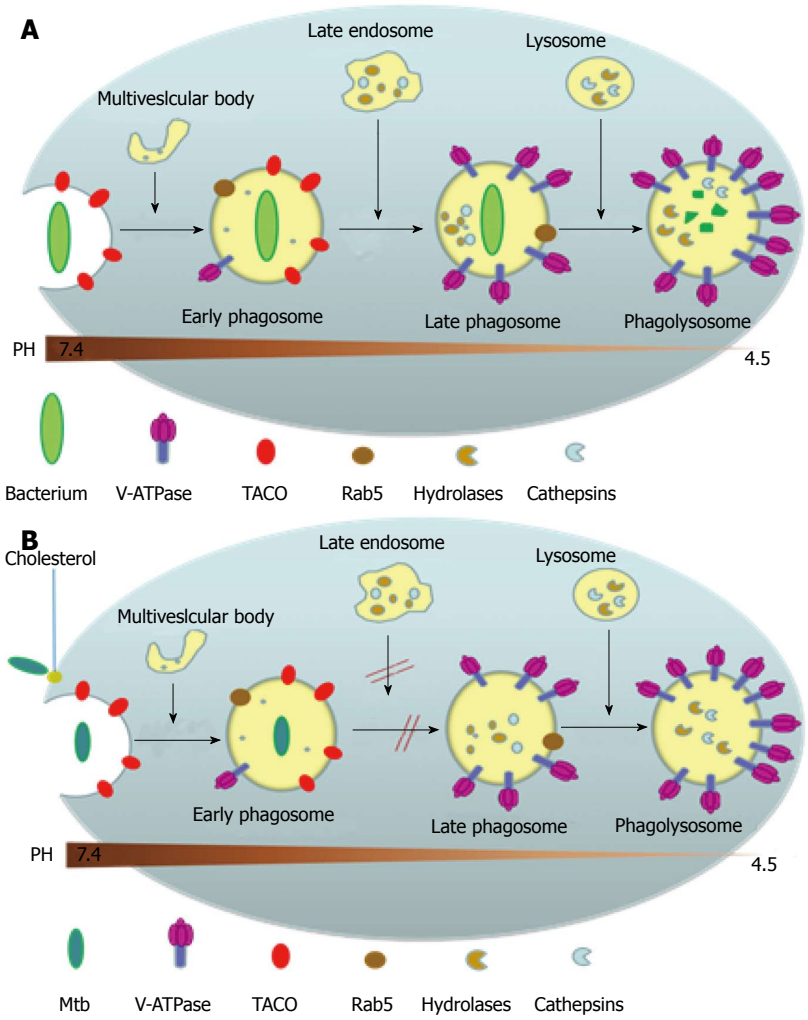

Figure 4 Phagosomal maturation or arrest following pathogen uptake (A) or *Mycobacterium tuberculosis* uptake (B), respectively. (A) shows that upon entering into the body, most non-pathogenic microbes are phagocytized by the macrophage into a phagosome which then goes on to mature by fusing with the vesicles of the endocytic pathway and to finally fuse with lysosomes. These phagosomes undergo acidification due to the presence of proton-ATPase molecules from vacuolar membranes and the lysosomes, and this increased level of acidification activates the lysosomally derived acid hydrolases, cathepsins and other enzymes, along with reactive oxygen and nitrogen intermediates, to destroy the pathogen. Phagocytosis also initially triggers the recruitment of TACO around the particle to be ingested, as a result of the latter's initial association with cell cortex microtubules, but is released prior to the lysosomal delivery of the bacteria; (B) Shows the effect of *M. tuberculosis* on phagosome maturation. Cholesterol serves as a docking site for the mycobacteria and its cell surface receptor there by facilitating its phagocytosis at cholesterol-rich regions. Cholesterol plays a crucial role in not only the entry of mycobacteria into macrophages but also mediates the phagososomal association of TACO (Coronin 1), a coat protein associated with cholesterol-rich regions which is actively retained on the phagosomal membrane housing the mycobacteria through a yet unknown mechanism, which prevents the degradation of the mycobacteria in the lysosomes. TACO: Tryptophan-aspartate containing coat protein.

Figure 5 The organization of the mce operons. The operon structures of the four mce operons with their constituent genes are shown. The grey arrows represent yrbE genes and the blue arrows represent the mce genes.

(Figure 6A). The YRBE permease contains five or six transmembrane segments outside the C-terminus and the orientation of the N-terminal transmembrane helix may be either cytoplasmic or outside (Figure 6B), suggesting a transmembrane transport role[47,48].

The MCE protein, including MCE1A, 3A and 4A, but not MCE2A, make up a patch of 275-564 amino acid residues, with the hydrophobic stretch at the N-terminal anchored in the membrane, after folding and modification[47], along with a 22 amino acid "invasion domain" near the C-terminal exposed outside the membrane[49,50] (Figure 6B). These characteristics are consistent with their cell surface location and proposed role in cell invasion and immunomodulation[51], however,

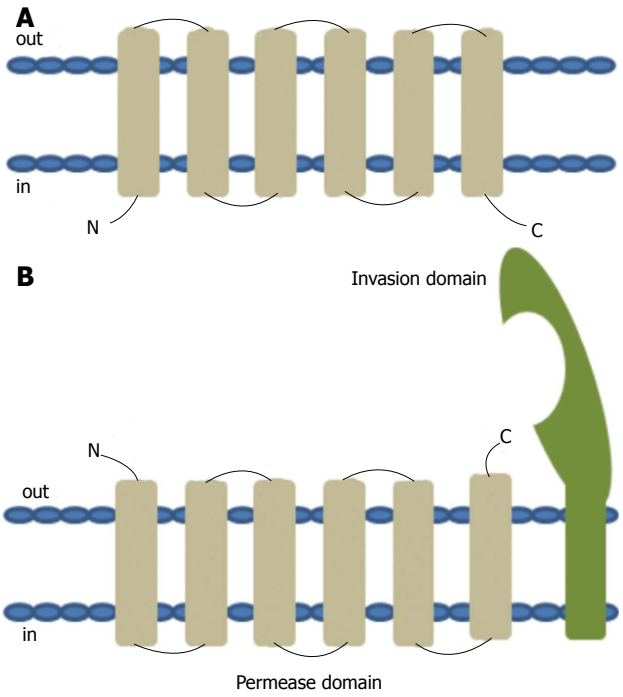

Figure 6 Topology of typical ATP binding cassette transmembrane permease (A) and the predicted topology of YRBE permease and MCE invasion domain (B). ATP binding cassette (ABC) transport systems in both prokaryotes and eukaryotes consists of four domains, two cytoplasmic ABC domains and two hydrophobic membrane spanning permease domains (A). Each of the membrane spanning domains typically contains 6 hydrophobic transmembrane segments anchored in the cell membrane with the C- and N-termini located towards the cytoplasmic side. ABC transporters in prokaryotes that function as importers also typically require additional extracytoplasmic helper proteins called 'substrate binding proteins' for function. Each of the mce operons contains two YRBE domains that have structural homology to the permease domain of the ABC transporters (B). Each YRBE permease contains five or six transmembrane segments with the C-terminal end located on the extracytoplasmic side and the N-terminal end being either cytoplasmic or extracellular. The MCE protein, MCE4A, consists of a patch of 400 amino acid residues, with a hydrophobic stretch anchored in the cell membrane and a 22-amino acid invasion domain exposed outside to the membrane, which is predicted to be structurally similar to the substrate binding domain of ABC importers.

the mechanism of interaction between YRBE and MCE proteins is not yet clear[52].

Phylogenetic studies have shown that the MCE proteins share between 30%-70% amino acid identity to their inter-operon counterparts but only 16%-26% identity with other MCE proteins encoded by the same operon. For example, there is 61% identity between MCE1A and MCE2A, however, it falls to 25% identity between MCE1B and MCE1C[53,54]. Because of the multiple *mce* operons in the genome, it is proposed that they may have redundant or time dependent activities. This possibility is supported by the temporal transcriptional expression differences during different stages of *in vitro* growth between *mce1* in comparison to *mce3* and *mce4*[55]. Differences are also seen with *in vivo* growing bacilli in that even though *mce1*, *mce3*, and *mce4* transcripts are detectable up to 24 wk post infection in rabbit lung tissue, only *mce4* transcript is detectable 16 wk post infection in guinea pig spleen[55]. The expression of *mce2* was not detected under any conditions tested[55].

Studies using *mce* operon deletion or disruption mutants of MTB have demonstrated varying effects with the different *mce* operons. Some studies have shown than disrupting *mce* operons lead to attenuation[56,57], while others have shown some degree of hypervirulence for the host MTB following the mutations[58,59]. Deletion of *mce* operons 3 and 4 attenuated MTB virulence in infected macrophages[60].

The *mce4A* gene is the first among the six *mce* genes in the *mce4* operon that is studied the most. Studies showed that the MCE4A protein is not only important for host cell invasion but also for survival of the MTB pathogen in human macrophages[61,62]. Individual *mce1*, *mce2*, *mce3*, and *mce4* mutants administered intranasally or intravenously in mice have shown to result in lower bacterial burdens and slower mortality of the infected mice, with *mce4* operon deletion showing the greatest effects on MTB virulence[56,60]. The route of infection was also shown to be having an effect on the attenuation results in one study[56]. Hypervirulence among *mce1* mutants have been demonstrated in two separate studies when administered intranasally, intravenously, or intraperitoneally[58,59]. The deletion mutants in all the above studies, however, were not identical in the nature of their deletions and had variations in their deletion sequences that possibly led to different polar effects on downstream genes, which may explain the discrepancy in the results of some of the deletion mutant studies.

Apart from its possible role in mediating host infection, it is thought that at least some of the MCE4 proteins form an outer membrane channel that mediates cholesterol entry into the cell, thereby enabling uptake by the mycobacterium of host lipids vital for its survival during the prolonged latent infection[63]. Transposon Site Hybridization studies have shown that certain *MTB* genes involved in the lipid metabolism was genetically linked to the *mce4* operon genes[60]. The *mce4* has been shown to encode a cholesterol transport system that enables the mycobacterium to derive both carbon and energy from the host membrane lipids and also for possibly generating sterol metabolites mediating its long-term survival within the macrophage[38], with the *mce4* expression progressively increasing as the latency phase advances[64].

ANTISENSE TECHNOLOGY IN BACTERIA

Hundreds of bacterial encoded short interfering RNAs (siRNA) have been reported over the past decade[65-67]. Majority of these siRNAs act by binding to their target mRNAs to bring about the repression. They fall into two major categories: some are encoded at locations farther away from the target gene (trans-acting) and others are encoded by DNA strand complementary to the target gene (cis-acting). The trans-acting siRNAs

generally share only limited complementarity with their target gene and thus is prone to have off target effects. Trans-acting siRNAs are by far the most characterized bacterial siRNAs and have been shown to usually require the chaperon protein Hfq for base pairing[68]. The cis-encoded siRNAs, or anti-sense RNAs, have perfect complementarity with their target gene and thus have more extensive and stronger base pairing. Among the reported bacterial antisense RNAs, some are short (siRNA), with around 100 nucleotides in length and are usually encoded by plasmids or bacteriophages, while some are chromosomally encoded and are longer, in some cases overlapping entire genes or corresponding to the 5′ or 3′ extension of the protein coding region of the mRNA. The 5′ untranslated region of the *mogR* mRNA in *Listeria monocytogenes* overlaps 3 genes on the opposite strand involved in the flagellar synthesis and serves as an example of chromosomally encoded long antisense RNAs[69]. The binding region of antisense RNA on the target mRNA can also vary and may be located in the 5′ end, 3′ end, the central region, or the entire coding region.

Antisense RNAs in the bacterial cell have been shown to repress many detriments to the cell such as transposons and toxic proteins. One of the first anti-sense RNA to be discovered in bacteria was the RNA-OUT of the transposon Tn10, which was shown to inhibit transposition by preventing the translation of transposase mRNA[70]. Antisense RNAs are also seen encoded opposite transposase genes in *Salmonella enterica*[71,72], *Caulobacter crescentus*[73], and *Listeria monocytogenes*[69]. Thus a critical role of bacterial antisense RNA, as in eukaryotes[74], appears to be the inhibition of transposition. There is increasing evidence that antisense RNAs downregulate the expression of toxic proteins[75,76]. It has been found that most of these repressed proteins are hydrophobic, small with less than 50 amino acids, and toxic at higher levels. An example of this tight repression of one such toxic protein is seen in *E. coli*, where the low levels of SymE protein is maintained by the LexA repressor of the save our souls response, the SymR antisense RNA and the Lon protease[77]. Some of the antisense RNAs to transposases and toxic genes, such as SymR, are expressed constitutively in the cell[77].

Studies have shown that antisense RNAs can positively and negatively regulate the expression of various transcriptional regulators and other metabolic and virulence proteins in bacterial systems. For example, The 109 nucleotide *GadY* antisense RNA of *E. coli* overlaps the intergenic region of the dicistronic *gadXW* mRNA which encodes two transcription regulators of the acid stress response genes and enhanced transcription of the *GadY* gene which leads to cleavage of *gadXW* mRNA into *gadX* and *gadW* transcripts, leading to positive regulation (increased expression) of those genes[78,79]. And on the other hand, in the nitrogen-fixing cyanobacterium *Anabaena sp.* PCC 7120, the approximately 2200 nucleotide *alr1690-α-furA* antisense RNA spans the entire *alr1690* coding region and extends through the gene encoding the ferric uptake transcriptional regulator, FurA, into its promoter and regulator regions and it helps decrease *furA* expression and translation, thereby acting as a negative regulator of iron absorption and nitrogen metabolism[80]. Similar regulatory RNAs controlling metabolic responses to environmental effects have been reported in many other bacterial systems[81-87].

Another recently discovered phenomenon is the antisense-mediated gene regulatory switch in the bacteria called the "excludon". This comprises a gene locus encoding an unusually long antisense RNA that spans divergent genes or operons with related or opposing functions. In such a regulatory system, the antisense RNA can inhibit the expression of one operon while functioning as an mRNA for the adjacent operon, there by acting as fine-tuning regulatory switches in bacteria[88].

Antisense RNA also regulates the expression of various structural and virulence factors in different bacteria. For instance, the 1200 nucleotide *AmgR* RNA which is encoded opposite the *Salmonella enterica mgtCBR* operon is responsible for the bacteria's virulence and survival in macrophages[89]. A number of other antisense RNAs modulating virulence and regulating host-pathogen interactions have been discovered in a variety of bacterial species over the years[90-97]. Antisense RNAs have also been found to impact other benign structural components including flagellar synthesis in *Rhizobium*[98], *H. pylori*[99], *L. monocytogenes*[69] and *S. enterica*[100].

The first complete experimental confirmation of short antisense RNAs in mycobacteria was published in 2009, which revealed 5 trans-acting and 4 cis-acting siRNAs in MTB H37Rv in the context of pH and oxidative stress[101]. By the end of 2013, a total of more than 200 endogenous antisense RNAs were experimentally identified in various mycobacteria, including 70 in MTB[102-110], 90 in *M. Bovis*[103,104,111], 9 in *M. avium*[112], and 44 in *M. smegmatis*[102-104,113]. From these recent studies, a stronger connection between mycobacterial pathogenesis and the levels of expression of the antisense RNAs have emerged but many new questions about their potential pathogenic *vs* housekeeping functions remain to be answered. The lack of identification of an Hfq homolog in mycobacteria prevents the current approach of coimmunoprecipitation, making the study of the role of antisense RNAs all the more difficult in this genus[102]. Pandey *et al*[114] have proposed an alternative protein, Rv2367, as a potential RNA chaperon in place of Hfq[114], however, studies are ongoing in this direction to find a functionally-equivalent chaperon or to get around this issue[113]. Also, the role of mycobacterial antisense RNAs in regulating transposition is not as clear as in other bacterial systems like *E. coli*.

Antisense RNAs can repress or modulate expression of target genes by a variety of mechanisms, including transcription interference, transcription attenuation,

degradation by endo- or exonucleases, or by blocking ribosome binding. When inducing transcription interference, the transcription of antisense RNA from one promoter hinders the RNA polymerase from either binding or extending the target gene transcript from the opposite strand[115,116]. This type of interference occurs only in Cis and does not involve base pairing. In transcription attenuation, the binding of the antisense RNA to the target RNA causes a conformational change creating a terminator structure in the mRNA leading to its premature termination of transcription[117]. Antisense RNAs can affect target mRNA stability by stimulating or inhibiting its degradation. When employing endo- or exonucleases for gene regulation, the antisense RNA, upon binding to its target mRNA, induces or blocks a ribonuclease target site within the mRNA or can indirectly block the binding of the ribonuclease at a distant site. In many bacterial systems, two major endoribonucleases have been identified, RNase Ⅲ which cleaves double stranded RNA into two with different stabilities than the original transcript[118-121], and RNase E, a component of the multi-protein degradasome complex which cleaves single stranded RNA and interacts with Hfq and globally affect mRNA stability[119,122]. It is not precisely clear how the antisense RNA modulates RNase E activity, but the proposed mechanisms include the donation of its 5' monophosphate to stimulate RNase E activity or physically block the RNase E recognition site by basepairing to downregulate activity[123]. Other ribonucleases in bacteria have also been identified with more specialized functions, including RNase G (a non-essential paralog of RNase E)[124,125], RNase P[126,127], RNase LS[128], RNase Z[129,130], RNase H[131], RNase J1/J2[132], and the recently characterized RNase Y[133]. Many of these ribonucleases have already been characterized in various Mycobacteria, including MTB and *M. smegmatis*[134-142]. Apart from these mechanisms, it has also been found that some antisense RNAs can physically block mRNA expression by binding to the Shine Dalgarno sequences of their target mRNA and prevent ribosome binding[66,77,80], or they may indirectly modulate expression by altering the target mRNA conformation[67]. Finally, antisense RNAs can exhibit dual functions by acting as mRNAs and antisense RNAs or cis- and trans- acting RNAs[78].

Two general mechanisms have been proposed for base-pairing in antisense RNAs. The first type is a single-step mechanism in which the antisense RNA makes initial contact with the target mRNA to form a duplex[143]. The second type is a multi-step system in which the initial duplex formed is stabilized by a protein, followed by the formation of the more stable complete duplex[144,145]. In many cases of base pairing for the antisense RNA, a stem-loop structure is found to be important, along with a "pyrimidine-uracil-any nucleotide-purine" U-turn motif[146].

Synthetic antisense RNAs are generally delivered either by expressing the antisense transcript from a gene introduced into the cell or by direct delivery of antisense oligonucleotides. Degradation of the antisense transcripts can be a problem for both these delivery approaches, however, this issue is mitigated by using sequences that form more stable hair-pin structures with paired ends[147]. In order to increase the stability and uptake, antisense RNAs have been modified in many ways, including the addition of peptide nucleic acid (PNA) or alternating 2'O-methyl to their backbones, switching ribose rings to morpholine rings (PMO), switching internucleoside bonds with phosphorothioates (PS-ODNs), or by conjugating cationic peptides to PNAs and PMOs[148-150].

Antisense technology using synthetic siRNAs have been used as a powerful tool in knocking down genes in prokaryotes (and also in eukaryotes), including hepatitis G virus[151], *influenza* virus[152], *picornavirus*[153], and *Trypanosoma brucei*[154]. When targeted to essential genes, siRNAs inhibit growth of *E. coli*[148,149,155,156], *S. enterica*[157], *Staphylococcus aureus*[158], *M. smegmatis*[159] and MTB[61,160]. Antisense RNAs have been successfully used for the study of bacterial growth and metabolism since this approach allows conditional knock down of target genes[161-165]. It has also been used for the study of various putative virulence factors in bacteria[166,167]. Antisense technology has helped in identifying new antibiotics[168,169], antibiotic targets[170,171], sensitizing bacteria to antibiotics[171-173], and to elucidate the mechanism of action of potential new drugs[171].

Molecular beacons are a newer class of antisense RNA tagged with a fluorophore/quencher pair and their use for *in vivo* detection and knockdown of mRNA is gaining popularity. Molecular beacon based short interfering RNA (MB siRNA) has recently been proven to be a powerful tool for therapeutic gene silencing because of its specificity, broad applicability, and high efficiency[174-176]. The on/off signals produced by the fluorophore/quencher pair depends on the conformational state of the MB (Figure 7). In the absence of the target mRNA, the stem brings the quencher in close proximity with the fluorophore and turns the fluorescence off with high quenching efficiency *via* fluorescence resonance energy transfer (FRET). In FRET, the energy from the donor chromophore is transferred to acceptor quencher nearby thus resulting in the absence of fluorescence. If the quencher and the fluorophore are far apart (following hybridization of the beacon to its target) then the quencher molecule will not be able to absorb the energy from the donor fluorophore. This would result in an increase in fluorescence. This technology has been used to detect mRNA expression in cells as well as for the detection and knockdown of telomerase expression in human breast cancer cells[177], *BMP4* mRNA in hedgehog signaling[178], aromatase mRNA in breast cancer cells[179], and for the detection and attenuation of *mce4a* mediated *M. smegmatis* infection in macrophages as discussed in section below[155,180].

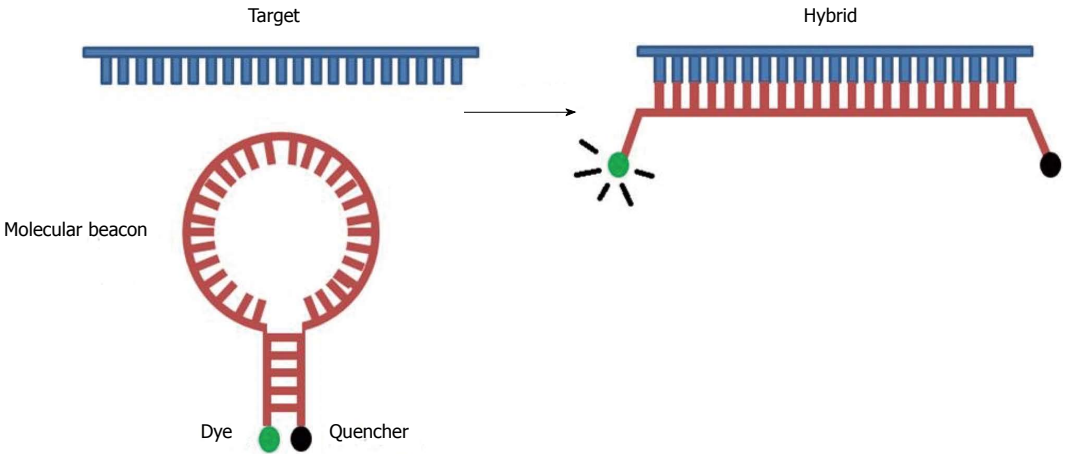

Figure 7 Molecular beacon technology. Molecular beacons are hairpin shaped antisense RNAs that does not fluoresce in the absence of mRNA binding (left) while the fluorescence increases when the beacon hybridizes with its target mRNA (right).

M. SMEGMATIS AS AN *IN VITRO* MODEL FOR STUDYING MYCOBACTERIAL INFECTION AND PERSISTENCE

The *mce* operons are widely seen throughout the genus *Mycobacterium* and a homolog of *mce4* has been confirmed in the mycobacterial species *M. smegmatis*[64,181]. Even though *M. smegmatis* is non-pathogenic, previous studies have shown that it can survive and multiply within macrophages in a pathogen-like manner by manipulating the host cell during initial stages by delaying phagosomal acidification and recruitment of V-ATPase[28,182], thus making it a suitable model to study *mce4 operon* mediated invasion and intracellular mycobacterial survival.

STUDIES IN OUR LAB ON THE ROLE OF MOLECULAR BEACONS IN DETECTING AND ATTENUATING MYCOBACTERIAL INFECTION IN MACROPHAGES

In order to determine the ability of a siRNA molecular beacon to detect and attenuate mycobacterial infection in macrophages, we first conducted experiments towards determining the most infective gene in the mce4 operon. Because of the slow growth rate of MTB and also due to the high degree of homology between *mce4* operons of mycobacteria[181], the *mce4* operon of the rapid growing *M. smegmatis* was selected for our studies. Using gene specific primers with the reverse primer for each set excluding the termination codon, each of the *mce4* genes, *mce4A, mce4B, mce4C, mce4D, and mce4F* were PCR amplified, cloned into the prokaryotic expression vector *pTrcHis2-TOPO* and stably expressed in *E. coli*. Western blot analyses with monoclonoal antibodies against c-myc and 6xHis showed that the MCE4 proteins were expressed in host *E.coli*. Next we conducted invasion assays in MCF7 breast cancer cells using *E. coli* clones expressing the *M. smegmatis* genes and the results showed that *mce4A-F* conferred virulence to its host *E.coli*. However, *mce4A* appeared to confer the earliest virulence to its host *E. coli* and the virulence was found to be sustained during the entire invasion period (72hr)[180]. We later, repeated the cloning experiments using the *mce4* operon genes of MTB, by PCR amplifying each of the *mce4A-F* (Figure 8), cloning into TOP10 *E.coli* using the prokaryotic expression vector *pTrcHis2-TOPO*, and performing invasion assay using MCF7 breast cancer cells. Our results showed that, as with *M. smegmatis*, the *mce4A* gene conferred the greatest degree of virulence to its host *E. coli* (Figure 9). Therefore, *mce4A* was selected as the target gene for designing a molecular beacon antisense RNA.

The *mce4A* antisense molecular beacon RNA was designed to have a stem-loop structure, with the nucleotides in the stem complementary to each other to form a 5-base pair double stranded stem and the loop consisting of 20 nucleotides that are complementary to a region of the target *mce4A* mRNA in *M. smegmatis*. Also, conjugated to the 5' and 3' ends of this molecular beacon are the fluorophore 5' TYE™ 665 and quencher Iowa Black RQ-SP (fluorophore quencher for 500-700 nm spectrum), respectively. This molecular beacon design combined both detection and therapeutic capabilities[177-179]. The rationale is that in the absence of the target *mce4A* mRNA, the molecular beacon remains in its hairpin form while in the presence of its target mRNA the 20 nucleotide loop will compete with the 5 nucleotide stem for hybridization to their target *mce4A mRNA* and the stem to its complementary pairs on the opposite ends of the target sequence. The hybridization potential of the loop to its target, based on the number of nucleotides within it (20 *vs* 5), will be greater than that of the strands for the stem. Hybridization of the loop to the *mce4A* mRNA will separate the fluorophore from the quencher thus inducing fluorescence (detection) and degradation (therapeutic) of the mRNA.

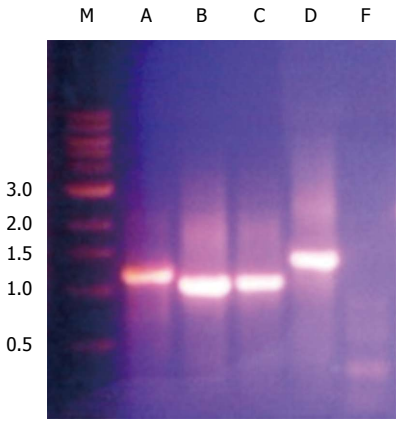

Figure 8 Polymerase chain reaction amplification of mce4A, mce4B, mce4C, mce4D and mce4F of *Mycobacterium tuberculosis*. *Mce4* operon genes were polymerase chain reaction amplified from *M. tuberculosis H37Rv* using gene specific primers and resolved on 1% agarose gel. The forward primer spanned the first 21 nucleotides from the beginning of the open reading frame and the reverse primer covered 21 nucleotides spanning the complementary strand to the 3' end of the gene. The termination codon was omitted so that the product, MCE4A-F, will be expressed with a 6XHis tag and a myc tag.

Figure 9 Mtb-MCE4 proteins confer virulence to *Escherichia coli*. *Escherichia coli-Mtbmce4* clones were used to infect 2 × 10^6 MCF7 epithelial cells at an MOI of 10:1 for 2 h. The level of infection was assessed by counting bacterial colony numbers at 24 , 48 and 72 h post-infection (n = 3).

Since the mycobacterium utilizes the product of *mce4A* for entry in to macrophages and for its survival using host cholesterol for carbon and energy source transported through the MCE4 transporters[41,45,62,183], the degradation of the *mce4A* mRNA will lead to its reduced survival. Our studies first tested the ability of the *mce4A* siRNA to detect its target *mce4A* mRNA in *M. smegmatis* and in macrophages infected with *M. smegmatis and* the results show that the molecular beacon siRNA detected its target in *M. smegmatis* and in macrophages infected with *M. smegmatis.* Thus, we were able to show that a molecular beacon can be designed against one of the *mce4* operon genes in *M. smegmatis* that facilitates the detection of mycobacterial infection in macrophages.

Tests were carried out to test the ability of this siRNA molecular beacon to not only detect but also attenuate mycobacterial infection in macrophages. Towards this end, we used a green fluorescent protein (GFP) expressing lentiviral vector, *piLenti*-siRNA-*GFP,* to successfully transduce and stably express the *mce4A* siRNA molecular beacon construct in macrophages infected with either *E. coli* expressing *mce4A* gene (*E. coli-4A*) or *M. smegmatis.* Using confocal imaging and Western blot analyses with anti-GFP antibodies, we were able to demonstrate stable expression of siRNA up to 48 h post transduction and infection using the GFP reporter.

After confirming the expression of the GFP protein by fluorescence imaging and Western blot analyses, invasion assay was carried out to assess the effect of *mce4A* siRNA on mycobacterial infection in macrophages. For this, differentiated U937 macrophages were transduced with *piLenti-siRNA-GFP* phage for 24hrs followed by infection with *E.coli-4A* or *M. smegmatis* for 3 h, and incubation for 0, 3, 6, 24, and 48 h, respectively. The cells were extensively washed and lysed in 0.1% Triton-X 100 lysis buffer and the lysates were plated on either LB agar containing 100 μg/mL ampicillin for *E. coli-4A* or 7H11 media for *M. smegmatis*. The degree of attenuation of *E. coli-4A* infection was compared between 3, 6, 24, and 48 h against that at 0 h baseline and was found to be 0%, 77%, 59.6%, and 99.7%, respectively. The degree of attenuation of *M. smegmatis* infection was compared between 3, 6, 24, and 48 h against that at 0 h baseline and was found to be 94.8%, 70.3%, 98.9%, and 93.4%, respectively. Thus, our results showed that the *mce4A* siRNA was able to significantly attenuate both *E. coli-4A* and *M. smegmatis* infection in macrophages[159].

Separate set of experiments were conducted to further test the hypothesis that the *mce4A* siRNA molecular beacon can attenuate the *mce4A* mRNA levels in *E. coli* expressing *mce4A* gene within infected macrophages. For this, reverse transcription polymerase chain reaction analysis was performed on lysates from differentiated U937 cells which were transduced with the *piLenti-siRNA-GFP* phage for 24 h, followed by infection with *E. coli-4A* for 3 h and incubation for 0, 3, 6, and 24 h. The cells were washed and lysed and the intracellular bacteria were isolated and washed at each time point of incubation. The bacterial sample from each of the time points were lysed and the mRNA was isolated and purified using DNAse 1 enzyme treatment. Reverse transcripts were generated using RTPCR and the cDNAs were amplified using gene specific primers for *M. smegmatis mce4A* and *E. coli 16S* rRNA gene as internal control. The degree of attenuation of *mce4A* mRNA levels was compared between 3, 6, and 24 h against that at 0hr and the results were found to be 0%, 81%, 40%, and 36%, respectively using densitometry gel analysis. Our results thus showed that *mce4A* siRNA was able to attenuate *mce4A* levels within infected

macrophages as opposed to *E. coli 16S* rRNA internal positive control and the degree of attenuation of *mce4A* mRNA levels in *E. coli-4A* was found to be significant.

Thus, we have successfully demonstrated that a molecular beacon can be designed against one of the *mce4* operon genes in *M. smegmatis* which can be used to both detect and attenuate mycobacterial infection in macrophages.

Antisense oligonucleotides, considered the pharmacology of the future[184], interact with their mRNA targets with greater specificity and binding affinity than traditional drugs to their protein targets. Recent advances have enhanced their hybridization to target mRNA, reduced their overall toxicity with decreased susceptibility to cellular nucleases. The lung provides an excellent target for direct antisense oligonucleotide delivery by inhalation, thereby achieving a bolus dose directly to the target site. Cationic lipids in the lung surfactants enhance oligonucleotides entry into cells[185]. Penetration of the inhaled oligonucleotides into deeper tissues of the lung has been established by autoradiogram, surgical dissection and receptor quantification studies[186]. Further studies to test the hypothesis that *mce4* siRNA respirable molecular beacons can localize and attenuate mycobacterial infection in pulmonary granulomas in animal models will take the fight against TB a long way in eradicating this versatile human pathogen.

CONCLUSION

The association of the *mce* operons, especially that of *mce4*, with mycobacterial invasion and latency is no longer considered casual and with strong evidences emerging over the recent years it can now be considered as a potent mediator of *MTB* infection and survival in its only human host. The mce invasion domain is equipped to mediate the entry and localization of the bacteria in the host macrophages at cholesterol rich regions creating cholesterol-associated protein coated phagosomes, thereby creating an ingenious mechanism for subverting the immune defenses. Another paradigm to the mycobacterial saga was added by the discovery that the MCE associated protein, YRBE4 transporters, in conjunction with the MCE4 domains, transport cholesterol into the cell for its energy and carbon needs, which then possibly generates metabolites that can further mediate its latency in the host. Strategies like identifying the level of infectivity of individual *mce* operon genes and designing efficacious drugs like molecular beacon siRNAs against *mce* targets can aid in the simultaneous detection and eradication of this elusive human pathogen.

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REVIEW

Computed tomography-based finite element analysis to assess fracture risk and osteoporosis treatment

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Abstract

Finite element analysis (FEA) is a computer technique of structural stress analysis and developed in engineering mechanics. FEA has developed to investigate structural behavior of human bones over the past 40 years. When the faster computers have acquired, better FEA, using 3-dimensional computed tomography (CT) has been developed. This CT-based finite element analysis (CT/FEA) has provided clinicians with useful data. In this review, the mechanism of CT/FEA, validation studies of CT/FEA to evaluate accuracy and reliability in human bones, and clinical application studies to assess fracture risk and effects of osteoporosis medication are overviewed.

Key words: Finite element analysis; Bone mechanics; Hip fracture; Osteoporosis; Vertebral fracture; Fracture risk

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Core tip: Finite element analysis (FEA) is a computer technique of structural stress analysis developed in engineering mechanics. With the faster computers, better FEA, using computed tomography (CT) has been developed. This CT-based finite element analysis (CT/FEA) has provided clinicians with useful data. In this review, the mechanism of CT/FEA, validation studies of CT/FEA to evaluate accuracy and reliability in human bones, and clinical application studies to assess fracture risk and osteoporosis treatment are overviewed.

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INTRODUCTION

Finite element (FE) method is a calculation technique

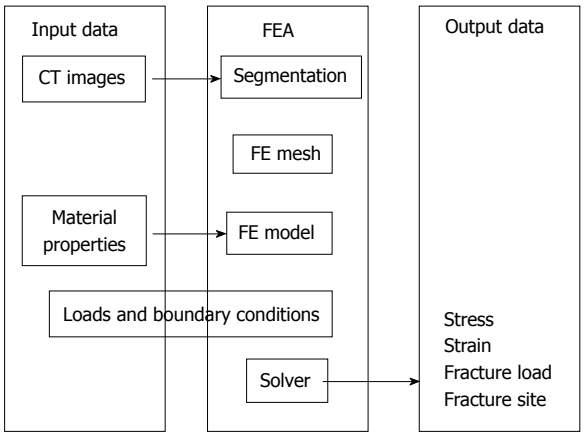

Figure 1 Steps involved in construction, analysis, and output data of computed tomography-based finite element analysis for human bones. FEA: Finite element analysis; CT: Computed tomography.

using computers to solve structural mechanics problems. With FE, approximate solutions of boundary value problems are solved. The concept of the FE is the subdivision of the mathematical complex structure into independent components of simple geometry. The number of components is finite, therefore the components are called "finite elements". The response of the mathematical model is considered to be discrete model obtained by connecting or assembling the collection of all elements. When examining artificial and natural systems such as bridge, building, airplane, and skeleton, the disconnection-assembly concept occurs. Calculation of structural mechanics problems using FE consists of dividing the structure into finite elements, and then each element is assigned by the element equations to the problem, followed by recombining all sets of element equations into the whole structure for the final calculation.

Finite element analysis (FEA) is a practical application of FE for performing structural analysis. FEA consists of using mesh generation techniques for dividing a complex structure into small simple elements and using software program with FE algorithm. FEA has been improved over the past 40 years and become an indispensable tool to assess structural mechanics of human tissues such as bones. In 1972, FEA was first applied in orthopedic biomechanics to calculate stress of human bones[1]. Since then, FEA has been widely applied to assess the human bone mechanics[2]. When the faster computers have been available, improved FEA and more advanced imaging modalities have been developed. The FEA using 3-dimensional (3D) computed tomography (CT) data was applied and was used to assess vertebral bone strength clinically in the early 1990s[3]. This so-called "computed tomography-based finite element analysis (CT/FEA)" has provided clinicians with useful data. Clinical applicability of CT/FEA of human bones might depend on accuracy, reliability, the ease of use, and usefulness. The purpose of this review article is to overview the mechanism of CT/FEA, validation studies of CT/FEA to evaluate accuracy and reliability in human bones, and clinical application studies to assess fracture risk and osteoporosis treatment.

MECHANISM OF CT/FEA FOR HUMAN BONES

There are three major stages, *i.e.*, pre-process, solution, and post-process, in CT/FEA for human bones[4]. The stages are summarized in Figure 1. The FE model is generated in the first pre-process stage and the problem of the model is solved by FE solver in the second solution stage. The solved results are analyzed in the post-process stage. The first step of CT/FEA is to acquire CT images of human bones, and then transfer the data to FE modeling system. After CT data are acquired, the region of interest (ROI) is segmented and mesh generation is proceeded. The quality and accuracy of the FE model is highly dependent on the ROI segmentation and mesh generation. A 3D image constructed with CT scanning is called voxel. In order to create FE model accurately and good quality, it is essential to define the anatomical bone shapes and segment the ROI accurately. After the ROI is segmented and the 3D bone geometry is obtained, mesh generation procedure can be done. There are several mesh generation techniques. In voxel based mesh generation technique, a pre-defined voxels from the CT scanning constitutes elements. In voxel based mesh, there is no surface or solid bodies. On the other hand, geometrical model with surface points is generated in structure based mesh procedure. Structure based mesh can generate more complex mesh and shell-solid mesh. In shell-solid mesh, component is modeled with two types of elements. Trabecular bone is modeled using solid elements. Outer surface of the trabecular bone, cortical bone or cortical shell is modeled using thin plate shell elements.

After FE model is generated, material properties of the each element in the model is assigned. The density of the each element is defined using the correlations between the Hounsfield unit (HU) value of the CT and the apparent density. The Young's modulus of the element is defined using the equations between the density and the mechanical properties of the bone. Regarding FE modeling of the human bone, there are two problems: (1) material properties of the cortical bone and the trabecular bone are very different; and (2) the material properties distribution within the cortical bone and the trabecular bone is not homogeneous. One of the methods to deal with the first problem is to use shell-solid mesh and model the cortical bone and trabecular bone using two types of elements. Cortical bone is constructed with "shell" elements. Trabecular bone is modeled using "solid" elements. It is essential to assure that assigned bone stiffness is connected to the assigned bone mineral density (BMD). The HU value of a voxel is connected to the bone stiffness. The HU values of a CT image are applied to define the

appropriate bone stiffness in the FE model.

An essential procedure to define material properties in FE model consists of calibrating the CT density, relating the voxel value to BMD, and accurately defining Young's modulus of the bone. For this purpose, a calibration phantom, which consists of known concentration of calcium hydroxyapatite, is used in CT image acquisition. By normalizing the HU values with the known concentration of the calibration phantom, an equation between CT density and BMD of the bone is established. It is shown that the apparent density and the mechanical properties of the bones are related with equations using power laws[5].

It is preferable that trabecular bone shows anisotropic mechanical behavior. Therefore, CT/FEA with anisotropic properties of the bones is assumed to be more accurate. In some studies, no significant difference was shown between the results by isotropic CT/FEA models and by CT/FEA orthotropic models[6]. But another study indicated a significant difference between the isotropic CT/FEA models and orthotropic CT/FEA models[7].

The final step is to apply the loads and boundary conditions in FE model. Boundary conditions defines that assigned nodes of the elements are limited in their degrees of freedom and are constrained. The constrained nodes are fixed or prescribed to move in a fixed amount. The applied loads work for deforming the element and producing internal stress. The solver works to solve the equilibrium between applied loads and internal material stress under the loads and boundary conditions. The location of the fixed element is defined by constraining the assigned nodes of the element completely. It is essential to apply the accurate loads and boundary conditions to fairly assess the mechanical behaviors in bone in daily activities.

CT/FEA SOLUTION AND CLINICAL APPLICATION

In order to apply CT/FEA in human bones, two important characteristics of bone have to be considered. First, bone is failed because of overstress or insufficient bone strength. Second, bone is adapting and remodeling by the applied loads and stresses, known as Wolff's law. Because of this bone tissue adaptation process, the mechanical properties of bone are changed.

In our increasingly aging societies, there are more patients with osteoporosis. Osteoporotic patients have much higher bone fracture risk due to insufficient bone strength. Fracture often occurs at hip, spine, humerus, and forearm. To determine whether pharmacological intervention for osteoporosis is needed, accurate fracture risk assessment is helpful. Traditionally, measurement of BMD by dual energy X-ray absorptiometry (DXA) has been the standard method for diagnosing osteoporosis. However, BMD itself is not able to predict the actual fracture in a reliable way. It has been reported that many patients which have normal BMD developed fractures due to osteoporosis[8]. The correlation between BMD and experimental fracture load was reported 45%-57%[9,10]. Recently, fracture risk assessment tool (FRAX) was developed to assist with clinical treatment decisions. FRAX was developed by the World Health Organization Collaborating Center for Metabolic Bone Diseases and calculates the 10-year probability of major osteoporosis related fractures (hip, spine, humerus, and forearm)[11-13]. FRAX can only be used in untreated patients. FRAX is useful for primary screening for osteoporosis and related fractures, but not useful for assessing therapeutic effects.

In CT/FEA, the stress and strain distributions are able to be assessed. Stress, strain, fracture load, and fracture site are assessed as a post-processing stage. Fracture is defined, and fracture load and fracture site are predicted with fracture criterion. For assessing the failure of engineering and biological materials, stress-based criteria or strain-based criteria have been used. Fracture criteria of bone include von Mises stress[14-16], Drucker-Prager stress[16-18], maximum principal strain[19], maximum principal stress[19], and minimum principal strain[17]. Some studies reported that bone fracture is regulated by strain and stress[20,21].

VALIDATION OF CT/FEA

After FE model with material properties is constructed, loads and boundary conditions are assigned, and CT/FEA solver solved the model simulation, the results are transformed into a useful output data, *i.e.*, stress, strain, fracture load, fracture site, to meet the investigation purpose. In addition, accuracy and validity of the CT/FEA should be shown through validation of the model. By comparing CT/FEA output data with the measured values in mechanical testing, CT/FEA can be validated and verified. It has been impossible to measure strains or stresses inside the trabecular bone structure with conventional methods. Therefore, strains at the bone surface are experimentally assessed, and it is considered that a validation of the surface strains substitutes for validation of the strains and stresses inside the bone. Strain gauges can measure strains at the site it attached. With strain gauges, strains at the some parts of specimen surface can be measured through mechanical testing. Recently, full-field strain measurement techniques that can measure surface strains throughout the specimen have been improved. Digital image correlation technique has been used for the measurement of surface strains during mechanical testing[22-24]. Digital image correlation has been successfully used for validation of the CT/FEA of the proximal femur[25].

For clinicians, fracture load, fracture pattern, and fracture site are the most useful data. There are some *ex-vivo* validation studies of CT/FEA analyzing the fracture load and then evaluate the accuracy by performing mechanical testing with human cadaveric

Table 1 Validation studies of the proximal femur

Ref.	Software	Variable	R^2	Slope
Cody *et al*[9]	EBE-PCG	One-legged stance fracture load	0.84	0.85
Keyak *et al*[14]	ABAQUS	Stance configuration fracture load	0.75	0.99
Keyak *et al*[14]	ABAQUS	Fall configuration fracture load	0.90	1.24
Keyak[27]	ABAQUS	Stance configuration fracture load	0.93	1.15
Bessho *et al*[18]	Mechanical Finder	Stance configuration fracture load	0.96	0.94
Bessho *et al*[18]	Mechanical Finder	Principal strain	0.93	0.91
Tanck *et al*[15]	MARC	Stance configuration fracture load	0.92	1.07
Derikx *et al*[16]	MSC (von-Mises criterion)	Stance configuration failure force	0.91	0.92
Derikx *et al*[16]	MSC (Drucker-Prager criterion)	Stance configuration failure force	0.91-0.94	0.93-1.01
Dragomir-Daescu *et al*[28]	ANSYS Mechanical APDL	Sideways fall fracture load	0.86	1.36

Used software of finite element analysis, validation variables, cross-validation R^2 values, and slope of the regression line are summarized.

Table 2 Validation studies of the spine

Ref.	Software	Variable	R^2	Slope
Silva *et al*[29]	ABAQUS	10 mm thick vertebral section yield load	0.91	0.86
Martin *et al*[30]	ADINA	Failure load	0.79	0.57
Crawford *et al*[31]	ABAQUS	Compressive strength	0.86	0.72
Imai *et al*[17]	Mechanical Finder	Failure load	0.96	0.88
Imai *et al*[17]	Mechanical Finder	Minimum principal strain	0.70	0.93
Kinzl *et al*[33]	ABAQUS	Apparent strength	0.92	1.02

specimens. For hip, validation studies indicated that CT/FEA might be able to predict femoral strength more than quantitative CT (QCT) or DXA[9] and was able to assess fracture site of the proximal femur[26]. Nonlinear CT/FEA showed more accurate predictions of fracture load at proximal femur[27]. Using shell-solid meshing, nonlinear CT/FEA analyzed fracture loads of the proximal femurs as well as principal strains of the surface of the femoral bones accurately[18]. Using Drucker-Prager yield criterion, CT/FEA could predict femoral bone strength and fracture site better than using von Mises yield criterion[16]. Validation studies of simulating a sideways fall on the hip revealed that CT/FEA predicted fracture loads and fracture patterns with a high degree of accuracy[28]. The validation studies of the proximal femur are summarized in Table 1.

Regarding spine, the cadaver studies have validated and verified that CT/FEA predicts failure loads and fracture patterns for vertebral sections with 10 mm thickness[29]. *Ex-vivo* validation studies of CT/FEA analyzing vertebral bones demonstrated that CT/FEA could predict vertebral compressive strength more accurate than BMD[30] and QCT[31]. Nonlinear CT/FEA using shell-solid meshing and Drucker-Prager yield criterion was able to predict distribution of minimum principal strains of the vertebra, vertebral strength, fracture pattern, and fracture site accurately[17,32]. Nonlinear FEA using high-resolution peripheral quantitative CT could predict vertebral strength well and the analyzed pressure distributions were qualitative agreement with the experiments measured by pressure sensitive films[33]. The validation studies of the spine are summarized in Table 2.

ASSESSMENT OF FRACTURE RISK AND OSTEOPOROSIS TREATMENT USING CT/FEA

Based on validation and verification with the cadaver studies, CT/FEA has been applied clinically in the assessment of fracture risk and osteoporosis treatment. In clinical data, the hip fracture risk index in discriminating hip fracture derived from CT/FEA was significantly better than total hip BMD and DXA-based structural engineering models[34]. The strength of the proximal femur varies depending on the specific force configuration.

In fall configuration, the force magnitudes and directions can be influenced by many biomechanical factors such as body weight, height, and position. The study to assess the relationship between femoral bone strength by nonlinear CT/FEA and incident hip fracture in multiple loading conditions, posterolateral loading in men and posterior loading in women were most strongly associated with incident hip fracture[35].

As for vertebral fracture, nonlinear CT/FEA had higher discriminatory power for vertebral fracture than lumbar spine BMD by DXA and volumetric BMD by QCT[36,37]. Consequently, CT/FEA has the potential to replace DXA and QCT in discriminating osteoporosis related fractures.

Regarding osteoporosis treatment, CT/FEA was useful for assessing teriparatide and alendronate medication effects at the lumbar spine[38]. In addition, a study using nonlinear CT/FEA clinically showed that vertebral compressive strength by CT/FEA was a significantly

better predictor for vertebral fracture than BMD, and was able to assess medication effects significantly earlier than BMD[36,37].

CONCLUSION

Osteoporosis related hip fractures and vertebral fractures have become a major social problem because the elderly population continues to increase. It is essential to assess fracture risk, start medication, and prevent fractures in the management and treatment of osteoporosis. In this article, the mechanism of CT/FEA, validation studies of CT/FEA for human bones, and clinical application studies to assess fracture risk and osteoporosis treatment are reviewed. CT/FEA accurately assesses bone strength and fracture site and is useful for assessing fracture risk and medication effects on osteoporosis. CT/FEA also assesses bone strength under various loading configurations normally seen in daily living activities[39]. With CT/FEA in the diagnosis and management of osteoporosis, patients and their clinicians are able to tailor a treatment plan according to a patient's specific clinical scenario.

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MINIREVIEWS

Multiplex planar microarrays for disease prognosis, diagnosis and theranosis

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Abstract

Advanced diagnostic methods and algorithms for immune disorders provide qualitative and quantitative multiplex measurement for pre-clinical prognostic and clinical diagnostic biomarkers specific for diseases. Choice of therapy is confirmed by modulating diagnostic efficacy of companion, theranotic drug concentrations. Assay methods identify, monitor and manage autoimmune diseases, or risk thereof, in subjects who have, or who are related to individuals with autoimmune disease. These same diagnostic protocols also integrate qualitative and quantitative assay test protocol designs for responder patient assessment, risk analysis and management of disease when integrating multiplex planar microarray diagnostic tests, patient theranostic companion diagnostic methods and test panels for simultaneous assessment and management of dysimmune and inflammatory disorders, autoimmunity, allergy and cancer. Proprietary assay methods are provided to identify, monitor and manage dysimmune conditions, or risk thereof, in subjects with pathological alterations in the immune system, or who are related to individuals with these conditions. The protocols can be used for confirmatory testing of subjects who exhibit symptoms of dysimmunity, as well as subjects who are apparently healthy and do not exhibit symptoms of altered immune function. The protocols also provide for methods of determining whether a subject has, is at risk for, or is a candidate for disease therapy, guided by companion diagnosis and immunosuppressive therapy, as well as therapeutic drug monitoring and theranostic testing of disease biomarkers in response to immunoabsorption therapy. The multiplex test panels provide the components that are integral for performing the methods to recognized clinical standards.

Key words: Simultaneous methods; Multiplex planar microarrays; Disease integrated panels; Theranosis; Prognosis; Diagnosis

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Core tip: Multiplex planar microarrays integrated for simultaneous, quantitative methods of prognosis, diagnosis, and theranosis provide a powerful technology for comparative measurements of changes in disease states and risk analysis, especially for autoimmune diseases.

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INTRODUCTION

Recent investigation of genomic and proteomic technologies have provided data for pathological conditions of immune function. Studies of human body fluids have confirmed that antigens, their specific antibodies as well as inflammatory mediators, to be diagnostic markers specific for inflammatory, allergic and autoimmune diseases. These proteomic studies have diagnostic and therapeutic benefits[1]. Serum auto-antibodies and inflammatory indicators are detectable even before onset of clinical symptoms and also during the course of systemic and organ-specific dysimmunity. Immune serum-derived markers become predictive biomarkers of disease in healthy subjects and markers of disease activity and severity in patients. New multiplex diagnostic technologies being introduced in laboratory medicine[2] allow the simultaneous detection of several different auto-antibodies and biomarker analytes for screening purposes in high-risk groups. Auto-antibodies with demonstrated diagnostic and predictive roles in organ-specific systemic disease are reviewed[3]. Cohort studies have shown that patients may have carried auto-antibodies and other markers of dysimmunity for extended periods of time, not diagnosed until discovery of other related clinical symptoms, as reported by Bizarro[4]. Serum auto-antibody assays have clinical utility in autoimmune diseases, including Addison's disease, celiac disease, Crohn's disease, biliary cirrhosis, Hashimoto's thyroiditis and type-1 diabetes. Dysimmunity is genetically and immunologically complex. Early diagnostic biomarker discovery is becoming increasingly more important for early diagnosis and early treatment in these diseases. Proteomic analysis of body fluids provides a non-invasive methodology for early diagnosis in many different disease settings. Proteomics-based approaches have made steady progress into biomarker discovery and understanding of autoimmune, allergic and cancerous diseases[5]. Traditional diagnosis, diagnostic proteomic methods including targeted antibody-based protein arrays, which have been particularly informative in the field of autoimmunity, are compared by Wu and Mohan[6].

Auto-antibodies are specific markers of autoimmune disease and humoral autoimmunity. Fluctuations in acute phase reactants, complement, cytokines, chemokines and growth factors suggest systemic and cell-mediated dysimmunity. Clinical understanding of autoimmune and allergic diseases includes analysis of auto and allo-antibody class IgG, IgM, IgA and IgE immuno-globulins. Multiplex, simultaneous identification measures different auto-antibodies in sera of patients suffering from autoimmune diseases.

The simultaneous measure of correlating analytes in reduced test volumes of patient's samples and test reagents, plus faster diagnosis, lowers assay costs and provides more diagnostic results in comprehensive serological profiles. Improved quantitative data is obtained by high-throughput techniques such as planar multiplex microarrays for antibody and analyte diagnostic profiling[7]. Recently collected data demonstrate more accurate analytical sensitivity and reproducibility. Aspects of multiplex assay sensitivity, specificity, co-efficient of variation and data interpretation are becoming standards of these new and promising clinical applications[8].

Gibson *et al*[9] present results to demonstrate that discrete, individual, clinical, laboratory or radiological parameters are limited to accurately diagnose or predict disease outcomes. Biomarker protocol panels which diagnose at an earlier time point, indicate early diagnosis to guide therapeutic strategies for dysimmune conditions towards more effective clinical management. There is a growing need for deeper understanding of multi-factorial immune disorders. Proteomic platforms offering a multiplex approach are more likely to reflect the complexity of dysimmune disease processes. Diagnomic approaches aid in early detection and are beneficial in guiding immune disorder treatment towards earlier disease prevention. Integrated panels facilitate these aims by offering comprehensive etio-pathogenesis test panels to screen asymptomatic and diagnostic symptomatic subjects, for a multitude of autoimmune, dysimmune and allergic conditions early in their disease course to monitor disease activity and severity for more effective treatment.

ANALYTICAL ATTRIBUTES

Autoimmune diseases include twelve currently recognized systemic, sixteen systemic vasculitis, eight idiopathic inflammatory myopathies, four immune-mediated systemic, thirteen endocrine and reproductive, five hepatobilliary and pancreatic, four gastrointestinal, four cutaneous, six cardiovascular and pulmonary, eleven neurological, four occular, five renal and eleven hematologic autoimmune diseases to be active in serious illnesses found in almost every human organ system[10]. The increasing incidence of autoimmune pathologies, *e.g.* rheumatoid arthritis (RA) in aging populations, is reported in the scientific literature by Lea *et al*[2]. Allergies are increasing in prevalence, impacting the burden of health care costs. Allergic rhinitis, asthma, atopic eczema and food allergy are known as co-causes of chronic ill health[11].

Antibodies to endogenous self-antigens or foreign allergens continue to be detected and recognized as reliable disease biomarkers for autoimmune and IgE - mediated allergic diseases. For example[2], a patient may have RA without experiencing symptomatic pain, which

can delay early diagnosis. Typically, autoimmune diseases develop slowly. In RA. Early prophylactic detection leads to early treatment at efficacious drug concentrations, with potential for differential diagnostic pre-symptomatic recovery, especially in patients who respond to drug therapy and/or in whom immunosuppressive treatment would be of benefit. The treatment method of choice may include specific therapeutic antibodies which differ from standard autoimmune treatment. New and more specific biomarkers indicative of various dysimmune pathologies continue to be recognized and accepted as providing disease confirmation, facilitating differential diagnosis, disease activity monitoring and developing therapy guided by companion diagnostics. This key attribute relates to companion diagnostics, wherein molecular assays that measure levels of proteins, genes or specific mutations provide a specific therapy by stratifying disease status, selecting the proper medication and tailoring dosages to the patient's specific needs. As a result of more accurate proteomic assessment, the FDA (United States) continues to set more demanding guidelines. Therapeutic products require validation based on diagnostic test to conform to label, safety and effectiveness claims. The increasing need for companion or personalized tests is demanded by subsets of treated, positive responders to possible side effects.

Detection of single, discrete biomarkers in isolated test outcomes results in a number of patients as misdiagnosed for autoimmune disease. Incorrect test outcomes are directly caused by patient samples that may contain unstable biomarker antigens, auto-antibodies, endogenous antibodies, patient sample proteins and antibody-antibody reactions. These analytes are often unstable or interfered with in patient's test samples as a direct result of time dependent storage leading to breakdown and cross-contamination, including effects of proteolysis, oxidation and protein interaction. Repeat testing of such stored samples confounds result of confirmatory tests. New diagnostic methods and algorithms for immune disorders need to provide robust, simultaneous and comprehensive, qualitative and quantitative multiplex measurement for prognostic and diagnostic biomarkers specific for autoimmune diseases, while modulating companion diagnostic efficacy of theranostic drug concentrations. Assay methods help to identify, monitor and manage autoimmune disease or risk thereof, in subjects who have an autoimmune disease, or are cohort related. Therefore, it is a benefit to provide integrated indicator measurement using multiplex planar microarray diagnostic tests, including test panels designed for responder patient theranosis assessment, risk analysis and management of disease.

DIAGNOSIS MANAGEMENT AND THERAPY

The panels provide for diagnosis, management and therapy of immune disorders and dysimmunity. In particular, they provide diagnostic tests, test panel design, methods of use and kits for simultaneous integration of qualitative and quantitative multiplex planar microarray prognostic and diagnostic tests, therapeutic drug concentration tests, drug efficacy tests, companion diagnostics tests and methods of use applied in specific test panels for simultaneous assessment, determination of risk and management of immune disorders. Specific disease panels include tests for genetic predisposition and also measure biomarker responses to treatment efficacy, such as drug dose concentration and drug pharmaceutical efficacy. This data will determine the optimal drug dosage for each patient as the drug level is maintained at the pharmaceutical efficacy level for that patient. Test panels are also designed to facilitate earlier detection of multifactorial etiologies, differential diagnosis from related disease phenotypes and detect disease activity indices such as remission, flare-up, relapse and life-threatening organ involvement. In assessing the risk of whether a patient has or, at some point in the future is prone to develop an autoimmune disease, wherein the method includes: (1) obtaining a sample from the subject; (2) multiplex array measurement of inflammatory indicators, antibodies of various classes to a plurality of different endogenous antigens or allergens in the patient's sample; (3) comparing the same analytes simultaneously measured in reference subjects, or to previous blood draws from the same patient, and or comparing reference patients having a similar immune disorder; and (4) identifying the risk that the patient has, or might develop an dysimmunity based on the comparison in step (3) above. Risk is present when levels of one or more of inflammatory mediators or antibodies to the different antigens or allergens are elevated in the subject's sample as compared to samples from reference patients with clinically normal functions, and/or are elevated in, or at about the same level in the subject's sample.

The patients samples as contemplated in the above method, can be biological body fluids, including peripheral blood, serum, plasma, cerebrospinal fluid, synovial fluid, bone marrow, saliva and urine samples.

In another method to qualify immune disorder patients, as eligible to receive disease-modifying therapy, the method comprises: (1) obtaining a sample from the immune disorder patient; (2) measuring the levels of each of a plurality of different analytes in the patient's sample; (3) comparing the levels with the level of the same analyte measured in samples from reference subjects with clinically normal function, or from reference subjects having disease corresponding immune disorder; and (4) identifying the immune disorder patient as eligible to receive disease-modifying therapy based on the comparison in step (3).

In another embodiment, the analysis relates to a method for identifying and selecting a patient with immune disorder for therapeutic drug monitoring, wherein the method comprises: (1) confirming clinical decision

making and diagnostic algorithms so that multiplex planar diagnomic microarray tests and disease panels can be implemented to monitor treatment efficacy; (2) test panels are integrated for simultaneous, qualitative and/or quantitative prognosis, diagnosis and theranosis that may include measurement of drug concentrations and efficacy; (3) test panels allow patients and physicians to make scientifically based medical, individualized decisions about their disease and its therapy; (4) theranostic panels are defined as biomarker tests that specify the dose and efficacy of therapeutics in reference to a patient's genotype, disease phenotype and serological profile, in conjunction with bioinformatics technologies that analyze and interpret human disease and drug response interaction; (5) antibody and biomarker levels are compared as indicators of disease predisposition, stage of development, aggressiveness, drug efficacy, dosing and toxicity; and (6) test panels form a core component of a new personal medicine to transform clinical practice into definitive, diagnostic science.

Disease test panels incorporating biomarkers that confirm pre-symptomatic disease indicators allow targeted disease therapy. Disease stratification lies in correlation of molecular heterogeneity of disease and heterogeneity of response to therapy. The targeted drug must be present and act on maintaining or deteriorating disease symptoms of the patient state to show efficacy. This target would therefore identify a biomarker to determine whether the patient is a likely responder for treatment with a specific therapy. For example, a multiplex planar microarray fluorescent immunoassay, provides quantitative measurement for anti-TNF drug (Tumor Necrosis Factor) concentration for Infliximab, which is used to treat patients with Crohn's disease and RA[2]. Infliximab Research Use Only assays provide 100% specificity. Results of this testing correctly reported the absence of the drug in healthy serum samples, but accurately reported drug levels when expected to be present, for example in RA patients responding to treatment. This novel microarray assay system detects and measures TNFu blocker drug concentrations at high sensitivity and specificity. Measuring the level of the TNF-alpha for anti-TNF-α therapy also provides evidence of treatment efficacy. Additional markers for [rheumatoid (RA)] disease activity, when measured simultaneously with anti-TNF-alpha drug and TNF-units would indicate disease remission.

Biomarker protocol panels identify patients responding to specific therapeutics, as disease progression and treatment efficacy is monitored. Simultaneous measurement of several disease parameters improves benefits of treatment for a patient. These biomarker panels identify the most effective drug for a specific patient. Retrospective drug efficacy analysis following clinical trial with subgroup analysis, clarifies response variations.

Diagnostics are rapidly developing for the management of diseases and emergence of personalized medicine and drugs. As the uniqueness of each individual disease process is confirmed, so is a growing assurance that particular drugs work better in subsets of patients that could not previously he clearly identified or segregated.

Clinical trials have confirmed that subsets of patients may derive no benefit or were harmed by the drug being investigated. A primary benefit for improved, marker-driven patient selection is to determine who is most likely to benefit from a proposed treatment and to exclude patients likely to be harmed by a drug. Advanced protocol test panels efficiently combine development of drugs with companion diagnostic tests.

Accordingly, multiplex analyses are combined in test protocol formats to detect one or more indicators for refining diagnosis and patient selection for treatments. Panels of biomarkers need to be monitored during clinical trials to confirm effective therapy, while simultaneously testing for and confirming drug side effects.

Biomarker based test panels have utility in all phases of clinical work to better understand and confirm disease onset, progression, symptoms and mechanisms of drug action in a population prior to drug approval by regulatory bodies.

Validation for disease status prediction, or dysimmunity, results from clinical accuracy, reproducibility, predictive value and diagnostic efficiency in detecting disease. The challenge in disease validation is variation of biomarker response observed in patient populations. As newly validated biomarkers transition to clinical diagnosis, it should progress through analytical validation including clinical performance and specifications. Clinical validation includes recognition of early stage disease, disorders and clinical parameters to clearly differentiate from similar disease phenotypes. Assays are then tested systematically to standard, reproducible, high-throughput formats.

Autoantibodies to endogenous autoimmune antigens can be identified in apparently healthy individuals. As components of the idiotypic network, these immune-globulins may multiply to cause patho-physiological impact[12]. Such auto-antibodies against endogenous autoimmune antigens can be monitored using multiplex diagnostic tests, methods and kits. The quantitative measurements of these auto-antibodies and other inflammatory mediators clinically evaluate subjects at risk of developing or currently suffering from autoimmune disease or other immune disorders and also be used to monitor efficacy of a treatment regimen. Particularly advantageous are confirmatory testing of patients who exhibit immune disorder symptoms, screening tests for apparently healthy patients with no symptoms of dysimmunity, who are likely to have symptoms in due course.

DIAGNOSTIC TEST METHODS

Integrated diagnostic test panels provide for assessment, risk analysis and management of immune disorders. In one embodiment, the panels provide means for risk stratification when developing an immune

disorder and in assessing the response to treatment. The methods entail protocols for assaying samples from the subject for panels of analytes, wherein the presence of elevated levels of one or more of the different analytes indicates the presence or risks of an immune disorder. These methods incorporating multiplex planar microarray diagnomic panels, integrate for methods of prognosis, diagnosis and theranostic companion diagnostics. Physical examination, medical history and histopathology, completes a differential diagnosis for autoimmune disease. Tests can be carried out on asymptomatic patients or patients having risk factors or symptoms of the disease. When screening and a patient tests positive for elevated levels of one set of analytes, the patient is assessed for one or more additional confirmatory tests.

A method of assessing risk of vascular pathology includes assaying a sample for auto-antigen and for auto-antibody specific for that antigen. A presence of auto-antigen or auto-antigen reactive to-antibody, indicates risk of autoimmune disease. Increasing levels of auto-antigen and auto-antibodies to autoimmune disease antigens elevates risk for autoimmune disease. In one embodiment, the analytes being assayed together with auto-antibodies, may contain as part of a test panel, biomarkers that are not specific to a particular autoimmune disease, but indicate disease activity and a generalized dysimmune or inflammatory condition. Moreover, at some stages of inflammation and dysimmunity, the tested analyte level may decrease rather than increase over time, to provide valuable diagnostic or prognostic information for evaluating risk of autoimmune disease for endogenous disease specific analytes, for a specific immune disorder or dysimmune condition.

THERAPY SELECTION

Selected test panels determine whether a patient at risk or diagnosed with an immune disorder, is a candidate for immuno-suppressive or immuno-absorption therapy. The method assays a sample for a plurality of analytes, wherein the presence of altered levels of at least one of the analytes or biomarkers indicates that dysimmunity may be contributing to the subject's immune or inflammatory disorder and the risks thereof. Therapy can be applied together with one or more other tests, physical examination and taking of medical history in accordance with standard practice for differential diagnosis of immune or inflammatory disorders.

Approving a patient with immune disorder to receive therapeutic immune-modulation considers measured levels of serum autoantibodies or inflammatory mediators using a panel protocol of different endogenous analytes, in comparison to known reference samples with clinically normal function and confirmed reference autoimmune disease. A patient would receive immunotherapy if levels of at least one analyte are higher or lower than normal. A patient with an elevated level of at least one analyte may also be treated with immune-suppressive therapy or immune-absorption therapy in accordance with standard practice.

MULTIPLEX PLANAR MICROARRAY PANEL ASSAYS

Multiplex planar microarray disease assay panel markers, when printed into a microarray multiplex planar assay promote the integration of differential diagnosis, evaluating disease severity and duration, disease prognosis and monitoring response to treatment including therapeutic monoclonal antibodies, on an individual patient basis[13]. Each panel is comprised of biomarkers and therapeutic biologicals that are used in the diagnosis, differential diagnosis, prognosis and theranostic monitoring of a patient in a general disease classification, *e.g.,* autoimmune diseases. Some analytes are part of the clinical criteria for defining a particular autoimmune disease such as Rheumatoid Factor in RA. Additionally, several phenotypes of other autoimmune disease may be co-existant, confounding a diagnosis based on clinical grounds alone. For example, arthritic disease includes RA, idiopathic arthritis, polyarthritis, osteoarthritis and mixed phenotypes (psoriatic arthropathy, arthritis with inflammatory bowel disease). Each may have particular markers and associated treatments. Their inclusion into one panel aids in the differential diagnosis (Bayes Theorem; decision tree analysis) in terms of positive predictive values and odds ratios for ruling in or out a particular phenotype based on clinical presentation and results from laboratory testing.

Prognostic markers to ascertain the disease duration, severity, activity (remission or relapse), organ system involvement and response to effective treatment are also included in the multiplex planar microarray disease panel assays. Where applicable, measurable biological drugs, especially therapeutic monoclonal antibodies, also constitute theranostic data to monitor how diagnostic markers fluctuate in response to treatment and to measure pharmacokinetics of the biologics.

Diagnomic marker analyte classes include autoantibodies, serum immune-globulins, immune complexes, therapeutic antibodies detected as IgG, IgM or IgA immunoglobulin classes, as well as antigen capture formats for acute phase reactants, cytokines, chemokines, growth factors and inflammatory mediators. Panels for Autoimmune Disease include Arthritis, Vasculitis, Nephritis Hematologic Disorders, Celiac and IBD and Thyroiditis.

CONCLUSION

These multiplex planar microarray disease panel quantitative assays diagnose particular autoimmune disease, such as RA, vasculitis, nephritis, celiac disease and

Crohns' disease. Similar panels can be designed for other inflammatory conditions and allergic diseases. Multiplex Planar Microarray Assays can also be used for differential diagnosis of a particular disease subtype in a spectrum of related diseases as in RA *vs* osteoarthritis or juvenile idiopathic arthritis. Disease prognosis in specific disease or spectrum of related diseases, with detection of disease activity, severity and organ system involvement markers, is an additional benefit. Multiplex Planar Microarray Assays can be applied to pharmacokinetic studies and therapeutic drug monitoring of biological drugs used to treat cancer and autoimmune diseases (*e.g.,* therapeutic monoclonal antibodies), as well as biomarkers that fluctuate in response to treatment.

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ORIGINAL ARTICLE

Retrospective Study

Eribulin for heavily pre-treated metastatic breast cancer patients

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Author contributions: Digklia A collected data; Voutsadakis IA conceived and designed the project, analyzed data and wrote the paper.

Institutional review board statement: As per our institution guidelines, no IRB approval is needed for retrospective reviews of patients whose care has directly involved or been supervised by an investigator.

Informed consent statement: Given that the study was retrospective in nature and the fact that treatments had been provided according to standards of care, no specific informed consents were needed or obtained from individual patients. Anonymity was guaranteed.

Conflict-of-interest statement: We have no conflicts of interest regarding this study.

Data sharing statement: No additional data are available.

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Abstract

AIM: To discuss treatment with eribulin in clinical practice outside a clinical trial.

METHODS: Archives of patients treated for metastatic breast cancer were reviewed and 21 patients treated with the new chemotherapeutic eribulin mesylate, a synthetic analog of a natural marine product, were identified. Information on patients' characteristics and treatment outcomes was extracted. Treatment with eribulin mesylate was initiated at the recommended dose of 1.4 mg/m^2 on days 1 and 8 of a 21-day cycle in 17 patients and at a decreased dose of 1.1 mg/m^2 on days 1 and 8 of a 21-day cycle in 4 patients due to comorbidities and frailty. Efficacy of the drug was evaluated using the revised Response Evaluation Criteria in Solid Tumors criteria. Progression-Free Survival and overall survival (OS) were calculated using the Kaplan-Meier method starting from the date of eribulin therapy initiation to the date of disease progression documentation or death, respectively.

RESULTS: The median age of patients at the time of eribulin mesylate treatment was 53 years (range 34-75). Sixteen patients had estrogen receptor (ER) and/or partial response (PR) positive disease and 5 had ER/PR negative disease (all triple negative). Eight patients had received 2 or 3 previous lines of chemotherapy

for metastatic disease and 13 patients had received 4 or more lines of treatment. The median number of cycles of eribulin received was 3 (range 1-16 years). All patients, except one, discontinued treatment due to progressive disease and one patient due to adverse effects. Six patients had a dose reduction due to side effects. All patients had progressed at the time of the report with a median time to progression of 3 mo (range 1 to 14 mo). Fifteen patients had died with a median OS of 7 mo (range 1-18 mo). Six patients were alive with a median follow-up of 13.5 mo (range 7 to 19 mo).

CONCLUSION: This series of patients confirms the activity of eribulin in a heavily pre-treated metastatic breast cancer population consistent with phase Ⅱ and Ⅲ trials.

Key words: Breast cancer; Metastatic; Eribulin; Case series; Retrospective; Treatment

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Core tip: This report discusses treatment with eribulin in clinical practice outside a clinical trial setting. It confirms the activity of eribulin in a heavily pre-treated metastatic breast cancer population.

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INTRODUCTION

Breast cancer is the most common female carcinoma and among the most frequent causes of cancer mortality in women worldwide[1,2]. Metastatic breast cancer is considered incurable with an estimated median survival of 2 to 3 years and only 1 in 4 to 5 patients alive at 5 years after diagnosis. Treatment with endocrine, cytotoxic or targeted therapies has prolonged survival and may improve or maintain the quality of life of patients living with metastatic disease. Despite the availability of several active agents, there is a need to develop additional drugs in order to increase the treatment options in metastatic breast cancer patients.

Eribulin (previously known as E7389) is a new chemotherapy drug and has been approved by regulatory authorities for the treatment of metastatic breast cancer[3]. It is a microtubule inhibitor and a synthetic analog of the natural product halichondrin B isolated from the marine sponge *Halichondria okadai*. Its mechanism of action is unique among microtubule poisons and involves binding to polymerized tubulin without affecting de-polymerization and promoting tubulin sequestration in non-functional polymers[4]. It produces cell cycle arrest in the G2-M phase *in vitro* in several human cancer cell lines, including p-glycoprotein-expressing and paclitaxel-resistant lines, when incubated in the presence of nanomolar concentrations of the drug. Clinical development has led to the approval of eribulin for use as monotherapy in metastatic breast cancer patients who have received at least two prior chemotherapeutic regimens including an anthracycline and a taxane. This is consistent with the view that sequential monotherapies are preferable to combination therapies in most clinical scenarios in metastatic breast cancer as a means of preserving tolerability without compromising efficacy.

This report presents retrospective data on this drug in patients treated in two centers outside of a clinical trial.

MATERIALS AND METHODS

Archives of all patients treated for metastatic breast cancer from 2011 to June 2014 were reviewed and 21 patients treated with eribulin were identified. Information on patients' characteristics and treatment outcomes was extracted. Data on response, progression-free survival (PFS) and overall survival (OS) were collected.

Treatment with eribulin mesylate was initiated at the recommended dose of 1.4 mg/m^2 on days 1 and 8 of a 21-day cycle in 17 patients and at a decreased dose of 1.1 mg/m^2 on days 1 and 8 of a 21-day cycle in 4 patients due to comorbidities and frailty. Tumor response was reported according to the revised Response Evaluation Criteria in Solid Tumors criteria[5]. Response rate was defined as the addition of complete response and partial response (PR). Disease control rate (DCR) was defined as the response rate plus the stable disease (SD) rate. PFS and OS are calculated by the Kaplan-Meier method starting from the date of eribulin therapy initiation to the date of disease progression documentation or death. Descriptive statistics parameters used in the study were performed with the aid of a non-commercial statistical calculation site (www.statpages.org).

Data collection and recording were conducted in compliance with the ethical requirements of our centers and patients' anonymity was guaranteed. Given that the study was retrospective in nature and the fact that treatments had been provided according to standards of care, no specific informed consents were obtained from individual patients.

RESULTS

The median age of patients at the time of eribulin treatment was 53 years (range 34-75 years) (Table 1). The median age at the time of breast cancer diagnosis was 47 years (range 30-73 years). Nineteen patients were diagnosed initially with localized disease and had received adjuvant treatments and 2 patients had metastatic disease at diagnosis. Sixteen patients had Estrogen Receptor (ER) and/or Progesterone Receptor

Table 1 Characteristics of patients and tumors in this series

	Median	Range
Age at diagnosis	47	30-73
Age at eribulin treatment	53	34-75
	Patients (n = 21)	%
Hormonal status		
ER/ PgR positive	16	76.5
ER/PgR / Her2 negative	5	23.8
Grade		
Ⅲ	12	57.2
Ⅱ	5	23.8
Unknown	4	19
Performance status		
0-1	13	61.9
2-3	8	38.1
Number of metastatic sites		
2	3	14.3
More than 2	18	85.7

ER: Estrogen Receptor; PgR: Progesterone Receptor; Her2: Her2/neu oncogene of the EGFR (Epidermal Growth Factor Receptor) family.

Table 2 Eribulin treatment and results

	Median	Range
Progression-free survival (mo)	3	1-14
Overall survival (patients who died)	7	1-18
Follow-up (mo, patients alive)	13.5	7-19
Number of eribulin cycles	3	1-16
	patients (n = 21)	%
Previous lines of treatment		
3 or less	8	38.1
More than 3	13	61.9
Reason for eribulin discontinuation		
Progression	20	95.2
Adverse effects	1	4.8

All patients in the series had progressed at the time of data collection. Fifteen patients had died at the time of data collection and 6 patients were still alive.

(PgR) positive disease and 5 had ER/PgR negative disease (all triple negative). Eight patients had received 2 or 3 previous lines of chemotherapy for metastatic disease and 13 patients had received 4 or more lines of treatment. The median number of previous lines of treatment was 4 (range 1-7). The median number of cycles of eribulin received was 3 (range 1-16). All patients had a heavy metastatic burden with two or more organs involved, including 5 patients with brain metastases and 10 patients with 4 or more metastatic organs.

All patients, except one, discontinued treatment due to progressive disease and one patient due to adverse effects (grade 3 asthenia) (Table 2). Six patients had a dose reduction due to adverse effects including febrile neutropenia, asthenia and peripheral neuropathy. Other grade 2 or 3 adverse effects observed included anemia in 2 patients and thrombocytopenia and ileus in one patient each.

Ten of 18 evaluable patients had a PR or SD for more than 3 mo as their best response resulting in a DCR of 55.6% (Table 3). Six of eight patients with 3 or less lines of previous therapies had a PR or SD resulting in a DCR of 75%, but some of the more heavily pre-treated patients (4 of 10, 40%) also benefited from eribulin treatment (Table 3). Three patients died before an evaluation of the effect of therapy could be performed. All patients had progressed at the time of the report with a median time to progression of 3 mo (range 1 to 14 mo) (Table 2). Fifteen patients had died with a median OS of 7 mo (range 1-18 mo). Six patients were alive with a median follow-up of 16 mo (range 7 to 19 mo). Kaplan-Meier PFS and OS curves are presented in Figures 1 and 2, respectively.

DISCUSSION

The new chemotherapeutic agent eribulin has shown activity in metastatic breast cancer and represents a new therapeutic option in this disease after failure of other therapies such as anthracyclines and taxanes[4].

Phase Ⅰ studies have established the optimal three-weekly dose to be 1.4 mg/m^2 on days 1 and 8 which has been carried out for further development[6]. Phase Ⅱ and Ⅲ studies have established the efficacy of eribulin in pre-treated metastatic breast cancer patients. Two phase Ⅲ trials have compared eribulin to either physician's choice or a capecitabine arm[7,8]. The first, the Eisai Metastatic Breast Cancer Study Assessing Physician's Choice versus E7389 open label, randomized, multicenter trial, included 762 patients who had received at least 2 previous lines of chemotherapy and were randomized in a 2:1 fashion to receive either eribulin or a treatment of their physician's choice[7]. The control arm was designed to reflect various practices across continents. Almost all patients had received anthracyclines and taxanes and three fourths had also received capecitabine before study entry. The study showed a statistically significant benefit in OS for patients receiving eribulin treatment with an OS of 13.1 mo *vs* 10.6 mo [7]. No PFS benefit was demonstrated in the intention-to-treat analysis as per the independent review. Median PFS was 3.7 mo in the eribulin arm. Hematological toxicities, asthenia/fatigue and peripheral neuropathy were the most common toxicities in the eribulin arm. The second phase Ⅲ trial was recently published and 1102 patients, who had previously received anthracyclines and taxanes, were randomized in a 1:1 ratio to eribulin or capecitabine[8]. The trial showed eribulin and capecitabine to be equally effective for both PFS and OS[8]. Median PFS was 4.1 mo in the eribulin arm in this study. Pooled results of these two randomized phase Ⅲ studies have confirmed a statistically significant OS benefit in the eribulin arm with a median OS of 15.2 mo *vs* 12.8 mo in the control arm[9].

Interestingly, in the above pooled study, an a posteriori sub-group analysis showed that the eribulin OS benefit was consistent in all sub-types with the triple negative patients obtaining a slightly greater benefit from eribulin compared to control treatments[9]. In a

Table 3 Clinical response rates of evaluable patients with metastatic breast cancer treated with eribulin *n* (%)

	All evaluable patients in the series (*n* = 18)	95%CI	3 or less previous lines of therapy (*n* = 8)	95%CI	More than 3 previous lines of therapy (*n* = 10)	95%CI
CR	0	0	0	0	0	0
PR	7 (38.9)	16.4-61.4	4 (50)	18.7-81.8	3 (30)	1.6-58.4
SD	3 (16.7)	0-33.9	2 (25)	0-55	1 (10)	0-28.6
PD	8 (44.4)	21.5-67.4	2 (25)	0-55	6 (60)	33.2-86.8

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

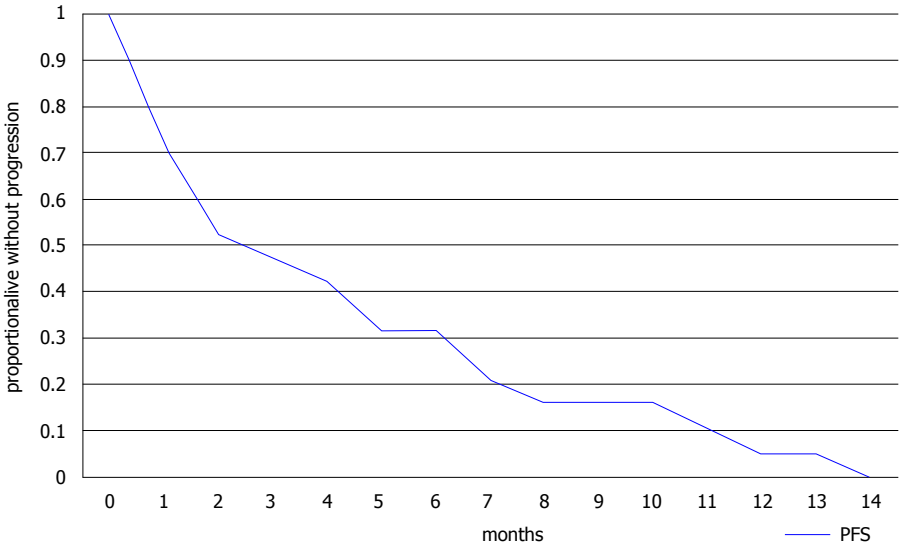

Figure 1 Kaplan-Meier Progression-Free Survival curve of the patients in this series. The Y axis depicts the percentage of patients alive without disease progression and the X axis represents months from the start of eribulin treatment. PFS: Progression-Free Survival.

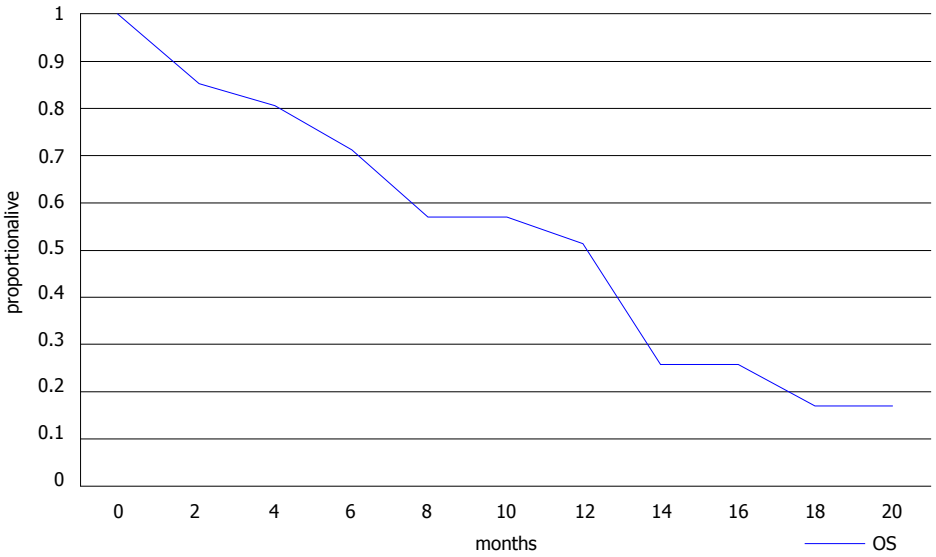

Figure 2 Kaplan-Meier curve of Overall Survival of the patients in this series. The Y axis depicts the percentage of patients alive and the X axis represents months from the start of eribulin treatment. OS: Overall survival.

retrospective study that included 133 patients, the clinical benefit of eribulin, especially in Her-2 negative patients, was confirmed[10]. In the first-line metastatic setting, a phase Ⅱ trial that included only Her-2 negative patients (both Hormone Receptor positive and triple negative) outlined the activity of eribulin in these patients which resulted in a median PFS of 6.8 mo[11].

Our small retrospective analysis confirmed that approximately 50% of patients were alive at 13 mo as shown by the Kaplan-Meier curve (Figure 2). Median

PFS was 3 mo and median OS was 7 mo in this heavily pre-treated population with a median of 4 previous lines of treatment (range 1 to 7). These results agree with those from the randomized trials and suggest that useful clinical activity can be expected in some patients who have received more than 3 previous lines of chemotherapy. The adverse effect profile of eribulin was also consistent with published data, with asthenia, neutropenia and peripheral neuropathy being the most common adverse effects. The patient population in our study was relatively young and only 3 patients were older than 65, however, studies have shown the feasibility of eribulin treatment in older patients[12].

Eribulin is added to the armamentarium of drugs with activity that may be used in metastatic breast cancer after anthracycline and taxane progression which may also include capecitabine[13], vinorelbine[14] and gemcitabine[15]. Nevertheless the short PFS highlights the need for more effective treatments or combinations.

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COMMENTS

Background

Eribulin is a synthetic analog of a natural marine product and is a new chemotherapeutic approved for the treatment of metastatic breast cancer.

Research frontiers

This report discusses treatment with eribulin in clinical practice outside a clinical trial. It confirms the efficacy of the drug in the day to day clinical setting.

Innovations and breakthroughs

This is a confirmatory report of the usefulness of eribulin mesylate for advanced metastatic breast cancer.

Applications

Eribulin is an additional option for the treatment of patients with metastatic breast cancer who have already received anthracyclines, taxanes and capecitabine.

Terminology

Eribulin (previously known as E7389): A chemotherapy drug which is a microtubule inhibitor and a synthetic analog of the natural product halichondrin B isolated from the marine sponge *Halichondria okadai*.

Peer-review

The paper described the observed outcomes of Eribulin treatment on a few breast cancer patients, it is a well written paper with a clear information on the outcomes of the treatment. It is a topic of interest to the journal's readers.

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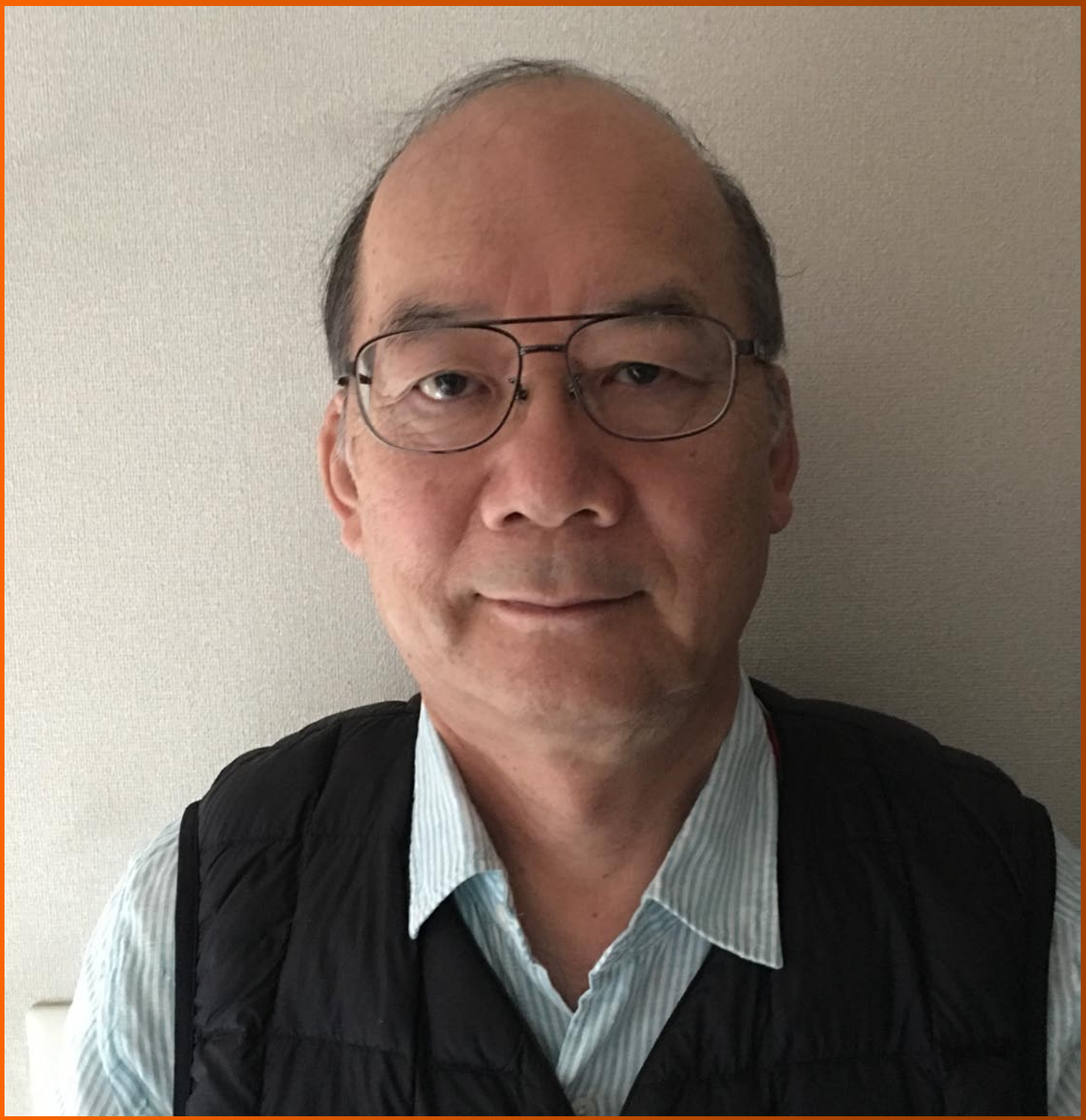

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EDITORIAL

Polycystins and mechanotransduction: From physiology to disease

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Abstract

Polycystins are key mechanosensor proteins able to respond to mechanical forces of external or internal origin. They are widely expressed in primary cilium and plasma membrane of several cell types including kidney, vascular endothelial and smooth muscle cells, osteoblasts and cardiac myocytes modulating their physiology. Interaction of polycystins with diverse ion channels, cell-cell and cell-extracellular matrix junctional proteins implicates them in the regulation of cell structure, mechanical force transmission and mechanotransduction. Their intracellular localization in endoplasmic reticulum further regulates subcellular trafficking and calcium homeostasis, finely-tuning overall cellular mechanosensitivity. Aberrant expression or genetic alterations of polycystins lead to severe structural and mechanosensing abnormalities including cyst formation, deregulated flow sensing, aneurysms, defective bone development and cancer progression, highlighting their vital role in human physiology.

Key words: Polycystins; Mechanotransduction; Kidney; Endothelium; Osteoblasts; Cancer

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Core tip: Polycystins are key regulators of mechano-sensation in several cell types including kidney, vascular endothelial and smooth muscle cells, osteoblasts and cardiac myocytes. Their expression in primary cilium, plasma membrane and endoplasmic reticulum, along with their ability to interact with diverse ion channels, cell-cell and cell-extracellular matrix junctional proteins renders polycystins as essential regulators of overall cellular mechanoresponse. Abnormal expression or genetic defects of polycystins result in severe structural and mechanosensing faults including cyst formation, deregulated flow sensing, aneurysms, defective bone development and cancer progression, highlighting their crucial role in human physiology.

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INTRODUCTION

Cellular mechanosensitivity plays fundamental role in cell viability and function, tissue development and maintenance of organs. Most cell types are able to respond to mechanical forces which provide them with a means of actively sensing and responding to mechanical properties such as topography and rigidity of the environment. Mechanical forces can be of external (such as acceleration, gravity, touch, stretch, sound) or of internal origin (breathing, fluid flow, blood pressure, osmotic pressure, heart contraction or any membrane deformation) and can vary from modest to high intensity depending on the cell type[1].

Mechanosensitivity constitutes a three-step process. It starts with detection of a mechanical stimulus by a cellular component, followed by mechanotransduction that converts the mechanical signal into a biophysical or biochemical signal, ending with the mechanoresponse during which signal sensation and transduction integrate over space and time[2].

Several mechanosensation models have been proposed based on the nature of the mechanosensor proteins[2-4]. Transmembrane proteins sense mechanical stimuli through changes in tension in their surrounding lipid bilayer ("bilayer tension model"[5]). Proteins involved in cell adhesion and maintenance of cell structure sense mechanical tension through binding with structural components that can transmit force from the intracellular or extracellular side or both ("Tethered protein model"). In this model interaction between the mechanosensor and proteins of the cell-cell junctions, extracellular matrix (ECM), focal adhesion points, microtubules or actin cytoskeleton has been reported. The way a protein responds to the applied force can also differ. A force applied to a mechanosensing protein can unfold it and expose cryptic peptides that can activate intracellular pathways and mechanotransduction[6] ("protein unfolding model"). Mechanical forces either exert a direct effect on the intrinsic activity of the mechanosensor proteins such as ion-channel gating, enzyme activity or ligand-receptor interactions ("mechanosensitive protein activity model"), or an indirect effect by activating a non-mechanosensitive protein leading to mechanotransduction ("adjacent mechanosensitive protein model"). The indirect activation can be through ligand release or through protein-protein interaction. All these models can also work in concert in order to form mechanosensitive complexes.

Mechanosensation is widely distributed in cellular compartments and involves the interaction of several protein complexes including adherent junctions, desmosomes, integrins, focal adhesion points, receptors and actin microtubules. Most of these proteins are connected to signaling pathways that involve cytosolic molecules, calcium signaling or transcription factors[7]. Their expression should match within time and space to the sensory function of the mechanosensor organ, while removal of the protein should annul the sensory response. Mutations that change protein function can modify the mechanosensing ability of the organ or cell. A heterologous expression of the mechanosensor protein in another cell type should lead to a mechanical response.

Interestingly, the protein family of polycystins has been shown to physically interact with most mechanosensing protein complexes mentioned above. Polycystins are implicated in renal flow sensing[8], vascular pressure and flow mechanosensation[9-11], blood-brain barrier mechanical injury[12], nodal flow sensing[13], skeletal development and osteoblast differentiation[14,15] as well as cancer progression[16].

POLYCYSTINS - STRUCTURE AND LOCALIZATION

Polycystins are large integral proteins, broadly expressed in human tissues including kidneys, blood vessels, heart, liver, pancreas, bone and brain. They are found localized in the primary cilium, at the plasma membrane and at endoplasmic reticulum (ER) where they associate and interact with numerous partners[17].

Polycystin 1 (PC1, 460 kDa) consists of 11 transmembrane segments, a short intracellular C-terminal region (200 amino acids) and an extracellular N-terminal part (3000 amino acids) which contains several protein motifs. These include a G protein-coupled receptor proteolytic site, two cysteine-flanked leucine-rich repeats, sixteen Ig-like domains and a C-lectin domain. The terminal intracellular C-region contains a coiled-coil domain (CC) as well as a G protein-binding site (G).

PC1 is found localized at the primary cilium and at the plasma membrane being involved in interactions between proteins and between proteins-carbohydrates. Scientific data support interaction of PC1 with many proteins localized at focal adhesion points, adherens junctions and desmosomes[18].

Polycystin 2 (PC2, TRPP2, 110 kDa) is composed of six transmembrane segments, an intracellular N-terminus which contains a ciliary sorting motif and an intracellular C-terminus with a calcium-binding EF domain, an ER retention domain and a CC domain. PC2 is located in the ER[19] while its translocation to the plasma membrane has been reported to require the presence of PC1[20].

PC2 belongs to the transient receptor potential (TRP) channel family proteins. It has been shown to interact with cytoskeletal proteins as well as other mechanosensitive ion channels in different cells, including potassium-selective stretch-activated potassium channels and non-selective cationic SAC channels.

PC1 and PC2 may interact through their CC domains located in the cytoplasmic C-termini forming an ion channel complex, as well as with many other partners in various subcellular localizations[20,21]. They are considered as important regulators of calcium homeostasis by affecting the resting cytosolic calcium

concentration, decreasing sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) expression and inhibiting the passive leakage of Ca^{2+} from the ER[22].

Since the original studies that identified polycystins and their gene mutations as a causative link to autosomal dominant polycystic kidney disease (ADPKD), considerable progress has been made in revealing the physiological functions of these proteins in multiple tissues, such as lung, kidney, cardiovascular, brain and bone[14].

POLYCYSTINS - MECHANOTRANSDUCTION IN THE KIDNEY

In the kidney, polycystins are detected at the cilia of renal epithelial cells[23]. PC1 though its extracellular domain is functions as mechanosensor detecting urine flow. Activation of PC1 leads to mechanotransduction by opening the PC2 channel allowing calcium entry and triggering intracellular calcium release in the ER through inositol 1,4,5-trisphosphate (IP3) or ryanodine receptors[8,24]. The mechanical properties of PC1 can be changed by osmolytes such as sorbitol or urea (a major urine component) and modulate mechanosensation[25]. Furthermore, at the primary cilium flow detection by PC1/PC2 complex induces proteolytic cleavage of the intracellular PC1 C-terminus (34 kDa fragment, called CTT), activation of signaling pathways [mammalian target of rapamycin (mTOR), Janus kinase/signal transducers and activators of transcription (JAK/STAT), Wingless-Int (Wnt)] and gene expression changes in order to get a mechanoresponse[26,27]. PC2 does not seem to need the presence of PC1 for its channel activity but it rather forms a heteromeric channel with TRP channel subfamily c member 1 (TRPC1)[27].

Flow sensing by primary cilium has been associated with increased intracellular calcium concentration being lost in PC1 or PC2-deficient cells and it has been proposed to result in cyst formation in polycystic kidneys[28].

Loss-of-function *Pkd1* or *Pkd2* gene mutations encoding PC1 and PC2, are responsible for ADPKD, the most common kidney disease, affecting almost 1 in 1000 individuals[29]. ADPKD clinical phenotype involves cysts presence in the kidney, pancreas, and liver along with severe cardiovascular defects. Arterial hypertension and intracranial aneurysms are often associated with this multisystem disease.

POLYCYSTINS - MECHANOTRANSDUCTION IN VASCULAR TISSUES

PC1 and PC2 expression has been observed in the plasma membrane and primary cilium of endothelial cells, proposed to transmit extracellular shear stress[30]. Shear stress-induced activation of PC2 has been demonstrated to increase the biosynthesis of intracellular NO leading to smooth muscle dilatation and flow-induced vascular relaxation[31].

In agreement, a previous study from our group using partial carotid stenosis to induce low shear stress *in vivo*, has shown upregulation of PC1 and PC2 in endothelium at the low shear stress area[11], implicating both proteins in blood flow alterations sensing. Since low shear stress conditions have been associated with atherosclerotic plaque development, a role of polycystins in atherosclerosis is possible.

Polycystins have been shown to interact with the two major calcium-release channels, IP3 receptors in epithelial cells and ryanodine receptors in cardiomyocytes. PC1 interacts with the IP3 receptors to reduce calcium levels[32]. Similarly, in the heart, PC2 interacts with the ryanodine receptor RyR2 *via* its C-terminus to modulate release of Ca^{2+} from the SR stores[33].

Notably, PC2 can form a channel with TRPC1, being activated in response to mechanical damage of blood-brain barrier endothelial cells by promoting Ca^{2+} influx and formation of actin stress fibers[12].

Finally, in vascular smooth muscle cells PC2 has been implicated in sensing pressure volume and in mesenteric and cerebral arteries in sensing myogenic tone[19,34].

POLYCYSTINS - MECHANOTRANSDUCTION IN OSTEOBLASTIC LINEAGE CELLS

In osteoblasts, the polycystin-primary cilia signaling complex has been attributed a mechanosensory role that regulates skeletogenesis and bone formation. Evaluation of the skeletal phenotype of *Pkd1*-deficient mice revealed PC1 implication in bone development and in the regulation of osteoblast function through intracellular calcium-dependent control of Runx2 expression. Furthermore, abnormal bone development and osteopenia was observed upon loss of *Pkd1* function in mice due to impaired osteoblast differentiation[14].

Another study of a mouse model with midpalatal suture expansion demonstrated that proliferation and differentiation of periosteal osteochondroprogenitor cells that were mechanically stimulated requires *Pkd1*[35]. This is in concert with a recent study from our group, exploring PC1 involvement in mechanical load (stretching)-induced signaling pathways in human pre-osteoblasts. In this study, PC1 was revealed as a major mechanosensor molecule in osteoblasts that modulates their differentiation and gene transcription through the calcineurin/nuclear factor of activated T-cells signaling pathway, thus controlling bone formation[15].

POLYCYSTINS INTERACT WITH CELL ADHESION AND CYTOSKELETAL PROTEINS TO TUNE OVERALL CELLULAR MECHANOSENSITIVITY

Polycystins ability to form multiprotein complexes with components of adherens junctions, focal adhesions, desmosomes and cytoskeleton suggests their critical role in regulating cell-ECM as well as cell-cell interactions.

Strong homophilic interactions between the Ig-like domain of PC1 with other PC1 and Ig domain-containing proteins of neighboring cells have been detected in the Madin-Darby canine kidney cells indicating their role in intercellular adhesion[30]. In cultures of renal epithelial cells, PC1 localizes at lateral cell junctions, involved in cell-cell interactions[30]. In addition, PC1 has been demonstrated to bind to focal adhesion proteins including integrins, pp125 focal adhesion kinase (pp125FAK), vinculin and paxillin which are required for cell adhesion to the ECM[36,37]. Of note, some of these associations have been lost in ADPKD epithelial cells[36].

In aortic smooth muscle cells, PC1 interacts with PC2 at the dense plaques which are involved in the interaction between the cytoskeleton, plasma membrane and ECM[38]. Regarding the components of adherens junctions, PC1 associates with E-cadherin and α-, β- and γ-catenins in a multiprotein complex required for maintenance of tissue structure and function[39,40]. In ADPKD renal epithelial cells, disruption of this multiprotein complex has been observed leading to depletion of PC1 and E-cadherin from the plasma membrane.

In desmosomes, the most abundant cell-cell junctions, PC1 has been found to directly bind though its cytoplasmic tail the CC motif of the intermediate filament proteins cytokeratins 8 and 18, desmin and vimentin[41]. In ADPKD cysts, these desmosomal elements are severely disoriented[42].

Finally, cytoskeletal proteins such as actin-binding proteins, cardiac troponin and tropomysin have been directly associated with PC2 channel activity upon stimulation by osmotic or hydrostatic pressure[43,44]. These numerous partners indicate that polycystins play a central role in the way cells adapt to their mechanical environment.

POLYCYSTINS - IMPLICATION IN CANCER PROGRESSION

All the aforementioned protein interactions that affect cell-cell and cell-ECM communication have been previously associated with cancer. Moreover, mechanical signals have been shown to regulate cancer cell interactions influencing decisive steps of invasion and metastasis. Recently, we have demonstrated the implication of PC1/PC2 in colorectal cancer progression. Overexpression of polycystins was associated with aggressive colorectal cancer phenotype *in vitro*. Clinical analyses revealed a correlation of elevated PC1 expression with poor recurrence-free survival, while aberrant PC2 levels was correlated with poor overall survival[16]. Several studies have also reported a connection between cancer proliferation, migration and metastasis with alterations in ion channels expression, and particularly with changes in TRP channel proteins[45]. More specifically, TRP channel subfamily M member 8 and TRP protein homologue (TRP6) were found highly expressed in prostate cancer where they correlate with histological grade. TRP channel subfamily M member 7 is implicated in proliferation and growth of breast cancer and head and neck tumor cells while TRP channel subfamily V member 4 and TRPC1 were associated with glioma growth[45]. Since some of these channels are known PC2 partners, it is highly likely the implication of polycystins in these malignancies and is currently under investigation.

CONCLUSION

Polycystins are envisioned as polymodal cellular sensors, critical regulators of cell structure integrity, cell communication, force transmission and subcellular trafficking in a broad range of cell types. Aberrant or defective expression of these proteins leads to abnormalities in calcium homeostasis and mechanosensation in major organs contributing to severe pathological conditions including ADPKD and cancer.

Future research should focus in elucidation of the mechanisms involved to integrate the information that arises from polycystin complexes into cellular functions. *In vitro* studies are needed to determine the kind of stimuli that trigger polycystins activation and the way that mechanical stimuli and ligand binding is sensed by PKD complexes. Are polycystins directly activated by mechanical stimuli or indirectly *via* activation of another protein partner? Animal models are required to define the functional consequences of PKD dysfunction in different cell types. How critical are polycystin-induced calcium signaling and enzymatic cascades for cellular growth and differentiation? Is there a specific role for multiple PKD complexes present at different locations in a single cell? How polycystins expression varies in different types of malignancy? Understanding the molecular mechanisms that underpin polycystins functions is urgently needed to identify novel and effective therapeutic schemes for the affected organs in the future.

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REVIEW

Endoplasmic reticulum stress-mediated pathways to both apoptosis and autophagy: Significance for melanoma treatment

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Abstract

Melanoma is the most aggressive form of skin cancer. Disrupted intracellular signaling pathways are responsible for melanoma's extraordinary resistance to current chemotherapeutic modalities. The pathophysiologic basis for resistance to both chemo- and radiation therapy is rooted in altered genetic and epigenetic mechanisms that, in turn, result in the impairing of cell death machinery and/or excessive activation of cell growth and survival-dependent pathways. Although most current melanoma therapies target mitochondrial dysregulation, there is increasing evidence that endoplasmic reticulum (ER) stress-associated pathways play a role in the potentiation, initiation and maintenance of cell death machinery and autophagy. This review focuses on the reliability of ER-associated pathways as therapeutic targets for melanoma treatment.

Key words: Melanoma; Endoplasmic reticulum; Apoptosis; Autophagy; Signaling pathways; Chemotherapy

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Core tip: This editorial describes the clinical validity of the endoplasmic reticulum (ER) as therapeutic target

for melanoma treatment. In addition, we highlight in this review the mechanistic role of ER stress in the modulation of both apoptosis and autophagy- associated pathways. Drugs that perturb ER function may represent an alternative approach for melanoma treatment. This paper reviews the pervious and current published studies on the reliability of ER-associated pathways as therapeutic targets for melanoma.

Hassan M, Selimovic D, Hannig M, Haikel Y, Brodell RT, Megahed M. Endoplasmic reticulum stress-mediated pathways to both apoptosis and autophagy: Significance for melanoma treatment. *World J Exp Med* 2015; 5(4): 206-217 Available from: URL: http://www.wjgnet.com/2220-315X/full/v5/i4/206.htm DOI: http://dx.doi.org/10.5493/wjem.v5.i4.206

INTRODUCTION

Although melanoma accounts for less than 5% of all skin cancers, it exhibits the highest mortality rate of all cutaneous tumors and its incidence is rapidly increasing[1]. The high mortality rate is the result of the propensity of metastatic dissemination throughout the body[2], and the development of resistance mechanisms that permit melanoma to evade normal immune surveillance mechanisms and the anti-tumor effects of chemotherapy[3]. Early detection and surgical excision of early stage disease offers the best hope of cure in patients with primary melanoma[4]. Even with new targeted therapies, the prognosis for advanced metastatic malignant melanoma is poor[5]. The available options for patients with advanced malignant melanoma patients provide limited therapeutic benefit with successful treatments often being measured in months of increased survival rather than years[6-8]. The potential to develop resistance mechanisms that counteract drug-induced apoptosis and evade host immunological responses is particularly devastating[9]. Accordingly, the replacement of single agent chemotherapy with targeted therapies is revolutionizing systemic therapy[10]. Besides the mechanistic role of mitochondrial damage-dependent pathways in the modulation of anti-cancer agent-induced apoptosis of tumor cells, anti-cancer agents can also improve killing efficiency *via* endoplasmic reticulum (ER) stress-dependent pathways[11-13]. While autophagy-mediated tumor death in response to anti-cancers is clinically relevant, these anti-cancer agents can also induce autophagy-mediated cytoprotective mechanisms[12,13], a pattern of tumor resistance to chemotherapy.

Metastatic melanoma demonstrates particularly poor response rates to single chemotherapeutic agents[14,15]. For instance, dacarbazine (DTIC) demonstrates no impact on survival, though it is considered to be one of the most effective agents that is used as standard therapy for the treatment of metastatic melanoma[16,17]. Other anticancer agents such as cisplatin, carmustine and the vinca alkaloids (*e.g.,* vindesine and vinblastine) fail to show any therapeutic advantage over DTIC[18], though several combination chemotherapy regimens demonstrate a modest increased response rate[19].

Melanoma's resistance to therapy is the results of an upregulation in pro-survival factors, which potentiate tumor maintenance and progression[20]. One of these factors is the inducible transcription factor NF-κB that is responsible for the regulation of the expression of genes related to apoptosis[21]. It is also, central to the development of tumor resistance to alkylating agents such as DTIC[22-24]. Accordingly, the inhibition of NF-κB pathway may improve the cytotoxic efficacy of alkylating agent-based therapy. To that end, preclinical studies *in vitro* and *in vivo* using human melanoma tumor models revealed that the therapeutic efficiency of DTIC or temozolomide is enhanced with the addition of the proteasome inhibitor, bortezomib[25,26].

Traditional mono- or multi-chemotherapy regimens are also associated with the development of significant adverse effects[27,28]. The development of new tumor types in these patients is attributed to the molecular action of the anticancer agents leading to the induction and/or destruction of aberrant signaling pathways.

The molecular action of chemotherapy in tumor cells is commonly associated with phenotypic alterations including cell death and survival-dependent mechanisms including apoptosis and autophagy[12,13].

Apoptosis and autophagy occur in normal cells. These are essential physiological mechanisms required for the maintenance of organismal and cellular homeostasis[29]. Current information about autophagy in melanoma focuses on autophagosome formation and/or autolysosome degradation in response to a variety of therapeutic agents using melanoma derived cell lines[13,30,31]. Chemotherapy induction of autophagy serves to protect melanoma cells from intendent chemotherapy-induced apoptosis. In fact, the induction of autophagy following the treatment of melanoma cells with bortezomib reduces bortezomib-induced apoptosis[13]. Similarly, the induction of autophagy by esomeprazole, a proton pump inhibitor, blocks melanoma cell death[32]. Based on this preclinical evidence, the modulation of autophagy-associated pathways offers a promising treatment strategy to increase treatment efficiency by overcoming melanoma resistance to chemotherapy.

The involvement of ER stress in the modulation of apoptotic mechanisms leading to melanoma cell death has been reported in several studies[12,13,33]. This may result from the induction of BH3 proteins such as Noxa and Puma leading to the inhibition of Bcl-2 localization at the ER membrane, alterations in the distribution of the calcium flux which produce ER stress[13,34].

Although ER stress and autophagy are capable of modulating each other in tumor tissues, their specific function is thought to be tumor type and stage-dependent[34-36]. The clinical potential of ER stress and/or autophagy-associated pathways as therapeutic

target for melanoma treatment has been reported in several studies[37-39]. For example, BRAF wild type (wt) melanoma is more sensitive to ER stress-based therapies than melanoma with hyperactivating BRAF mutations[40]. The frequency of BRAF mutation seems to be associated with elevated levels of autophagy in melanoma. Accordingly, ER stress-induced apoptosis of melanoma cells harboring oncogenic BRAF is lower than those observed in BRAF wt melanoma cells[40-42]. Inhibition of autophagy is a good strategy to sensitize BRAF wt melanoma cells to ER stress-mediated apoptosis. In addition, the development of anti-cancer agents based on the enhancement or suppression of these processes may be relevant therapeutic strategies[38,43,44].

Tumor resistance or response to available therapeutic modalities depends on the balance between apoptosis and autophagy-associated mechanisms[45,46]. Although the development of the most available therapeutic approaches focuses on the excessive activation of mitochondrial dysregulation-dependent pathways leading to apoptosis, there is increasing evidence that ER stress-associated pathways represent an important therapeutic target for melanoma treatment[13,47]. Thus, the development of anti-cancer agents with ability to trigger the intrinsic activation of ER stress/unfolded protein response (UPR)-associated pathways may offer a novel therapeutic strategy for tumor treatment. UPR is mediated in response to the enhancement of protein synthesis through the activation of mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway that, in turn, induces cell proliferation, a mechanism that can block ER stress-induced apoptosis[48]. Thus, ER stress-dependent pathways have been proposed to represent a new therapeutic target for melanoma treatment[10,49]. Accordingly, the inhibition of oncogenic BRAF (V600E) and/or MEK-attenuated activation of inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) signaling of the UPR in melanoma cells may sensitize melanoma cells to apoptosis. Our work focuses on the reliability of ER stress-dependent pathways as a therapeutic target for melanoma treatment.

FUNCTION OF ER IN NORMAL AND TUMOR CELLS

ER is a network of tubules and flattened sacs comprising rough and smooth regions that differ in their structure and function[50]. The rough ER is characterized by the existence of ribosomes attached to the cytoplasmic side of the membrane, whereas the smooth ER lack these ribosomes[50]. ER plays a crucial role in normal cellular functioning, by processing of post-translational modification and folding of secretory and membrane proteins. These secretory and membrane proteins are synthesized along the membrane of the rough ER and subsequently are passed onto the Golgi apparatus, where they undergo further post-translational modifications by the attachment of lipid and glucose moieties in a lipidation and glycosylation-dependent manner, respectively[51]. The ability of ER to correctly fold nascent proteins depends on chaperone proteins that, under normal physiological condition, are in excess in the ER lumen[52]. The function of most chaperone proteins is known to be Ca^{2+}-dependent[53]. ER contains a high concentration of Ca^{2+} and is the only cellular organelle that plays an essential role in intracellular Ca^{2+} homeostasis[54]. Thus, the escalation of intracellular calcium into the cytoplasm is a signal for pathophysiological alteration of the cells. This pathological phenomenon results from ER stress in response to externally physical or chemical stressors, such as radiation and toxins[55].

ER function is critical for the regulation of many aspects of cell physiology, such as vesicle trafficking, lipid and membrane biogenesis as well as protein targeting and secretion. Normal and tumor cells react rapidly to ER stress *via* mechanisms mediated by a set of ER stress-associated pathways. The regulation of these pathways is thought to be the consequence of the perturbations in ER function, such as the accumulation of unfolded or misfolded proteins, as well as the accumulation of ER lipid, glycolipid imbalances, or alteration in the ionic or redox conditions in the lumen of ER[56,57]. Three distinct signaling pathways have been identified as ER stress-dependent pathways, namely protein kinase RNA-like endoplasmic reticulum kinase (PERK), ATF6, and IRE1 pathways. The primary purpose of these pathways is implicated to promote cell survival by mechanisms mediated through the reduction of the misfolded protein[58]. Figure 1 outlines the ER stress-associated pathways in normal and tumor cells.

INDUCTION OF ER STRESS-ASSOCIATED PATHWAYS BY ANTI-CANCER AGENTS

Dysregulation of ER homeostasis is a primary pathophysiological mechanism responsible for the initiation of an ER stress response that leads to the development of a number of human diseases including cancer[59]. The induction of ER stress by anti-cancer agents and other stimuli has been reported in several studies. The anti-cancer agent's bortezomib, vinblastine and taxol trigger ER stress in melanoma cells[13,60,61]. Similarly, caffeic acid phenethyl ester, the BH3 mimetic obatoclax and the Abbott Compound ABT-737 have been reported to induce ER stress in melanoma[33,62]. Interestingly, the induction of ER stress in melanoma cells by these agents is correlated with the deregulation of ER stress associated pathways including eukaryotic translation initiation factor 2α (eIF2α) and PERK.

ER stress induced activation of PERK leads to the phosphorylation of the eIF2α that inhibits the translation and subsequently triggers cell cycle arrest[63]. CHOP (C/EBP homology protein) is downstream of PERK-

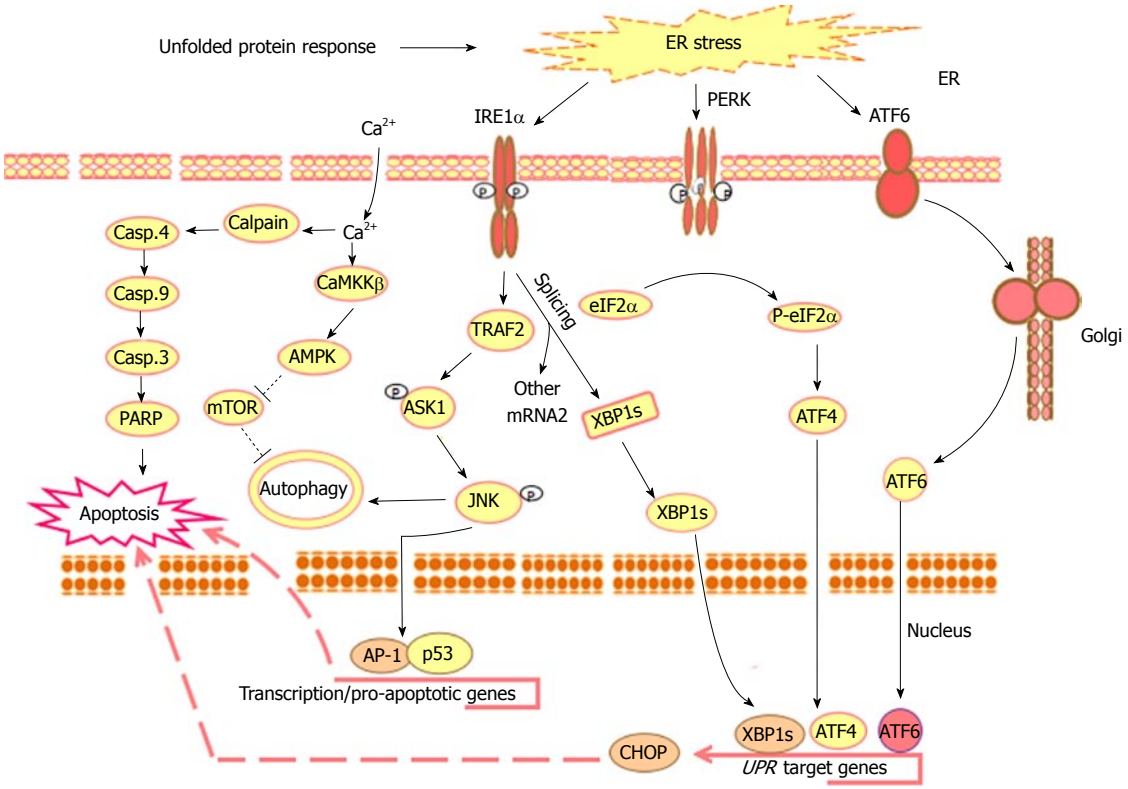

Figure 1 Outline of the main unfolded protein response-mediated mechanisms in response to the molecular action of anti-cancer agents. Upon the accumulation of misfolded proteins in the ER lumen, chaperones, the ER stress sensors PERK, IRE1α and ATF6 become active. The phosphorylation of PERK allows it to assemble in a homo-dimer to form an active form that, in turn, results in the phosphorylation of eukaryotic initiation factor2α (eIF2α) to initiate UPR downstream response leading to reduction of the protein overload to ER by the suppression of the translation and activation of ATF4 together with ER stress associated transcription factors such as, CHOP, PERK, IRE1 and ATF6. Activated PERK phosphorylates the translation initiation factor eIF2α to decrease the protein synthesis and enhance stress-inducible messages, such as ATF4. During ER stress the ATF6 traffics to the Golgi apparatus, where it is cleaved by S1P/S2P proteases. The cleavage of ATF6 from the Golgi membrane facilitates its localization to the nucleus, where it enhances the transcriptional up-regulation of UPR target genes leading to apoptosis. Whereas, activated IRE1α is implicated into several functions: One of these functions is essential to drive the splice mechanism of the XBP-1 mRNA to allow the translation of mature XBP-1 protein that, in turn, functions as a transcription factor to promote the transcription of UPR target genes such as CHOP leading to apoptosis; the other function of the activated IRE1α is to recruit TRAF2 that subsequently mediates the phosphorylation of ASK1 and the activation of its downstream JNK leading to the activation of the transcription factors AP-1 and p53 that are essential for the transcription of the pro-apoptotic genes and genes implicated in the processes of autophagsome formation and autophagy. UPR results also in intracellular calcium release leading to cell death *via* Calpain/Caspase-4, Caspase-9, caspase-3 and PARP axis or autophagy *via* CaMKKβ/AMPK axis leading to the inhibition of mTOR and subsequently autophagy. ER: Endoplasmic reticulum; PERK: Protein kinase RNA-like endoplasmic reticulum kinase; IRE1α: Inositol-requiring protein 1; ATF6: Activating transcription factor 6; UPR: Unfolded protein response; XBP-1: X-box binding protein 1; CHOP: CCAAT/enhancer-binding protein (C/EBP) homologous protein; TRAF2: TNF receptor-associated factor 2; ASK1: Apoptosis signal regulating kinase 1; JNK: C-jun-N-terminal kinase; PARP: Poly (ADP-ribose) polymerase; CaMKKβ: Calmodulin-dependent protein kinase kinase-β; AMPK: AMP-activated protein kinase; mTOR: Mammalian target of rapamycin.

eIF2α-ATF4 and involved in the regulation of the apoptotic proteins of the Bcl-2 family members[64]. ER stress-induced activation of IRE1α is responsible for the regulation of the transcription factor XBP1[65]. Once ER stress is initiated, the conversion of unspliced XBP1 mRNA to mature mRNA is mediated permitting the translation and further modification of this protein to operate as an active transcription factor[66]. The activation of the transcription factor XBP1 is essential for the induction of the transcription of ER-related genes that, in turn, mediate the disposal of unfolded proteins[67]. Although this panel of responses is mainly implicated in restoring ER homeostasis, sustained ER stress is essential for the promotion of apoptosis[68,69]. More importantly, it has been demonstrated that ER stress-associated pathways are involved in the modulation of the anti-cancer agent-induced apoptosis of tumor cells, particularly, in melanoma[13]. In recent years, we and others uncovered the mechanistic role of ER stress-associated pathways such as PERK-ATF4-CHOP/Bim and IRE1α-ASK1-JNK-AP-1/HSF1-HSP70, in the modulation of anti-cancer agent-induced apoptosis of melanoma cells[12,13]. More importantly, we demonstrated that Noxa-induced ER stress triggers apoptosis of melanoma cells *via* mechanism mediated by ASK1-JNK/p38 axis[58,70]. Also, apoptosis related protein-2 (APR-2)-induced ER-stress drives apoptosis of melanoma cells *via* mechanism mediated by three parallel pathways, namely IRE1α/tumor necrosis factor receptor-associated factor 2 (TRAF2)-ASK1-JNK/Cytochrome c/caspase-9/caspase-3/PARP, Calpain-caspase-4-/caspase-9/caspase-3/PARP, and PERK-ATF4-CHOP/Bim[58]. Furthermore, bortezomib/vinblastine-induced ER stress in melanoma cells is essential for the induction of cell survival *via* autophagy-dependent pathways including, IRE1α-ASK1-JNK-AP-1/HSF1-

HSP70 axis. More importantly, in our laboratory, we demonstrated that the inhibition of IRE1α-ASK1-JNK-AP-1/HSF1-HSP70 pathways synergistically enhance bortezomib or vinblastine-induced apoptosis of melanoma cells[12,13].

ER stress-mediated pathways to apoptosis in melanoma

It is established that the primary function of ER stress is to restore normal ER homeostasis and to engage cytoprotective mechanisms to counteract or mediate both intra- and extracellular-induced alterations[71]. Therefore, if the induced ER stress is strong or persistent, the ER enhanced dysfunction becomes irreversible and consequently triggers cell death machinery to initiate apoptosis. Thus, the destruction of the ER stress-dependent pathways that are essential for the modulation of the cytoprotective machinery by small molecule-inhibitors would be expected to trigger apoptosis of tumor cells. In addition, the enhancement of key components leading to excessive activation of apoptotic pathways, such as the mammalian IRE1α, could impact the regulation of kinases such as ASK1[62]. The activation of the pro-apoptotic kinase ASK1, the upstream kinase of the JNK pathway, is essential for the regulation of ER stress-induced apoptosis of melanoma in response to chemotherapeutic agents such as vinblastine[12], as well as in response to pro-apoptotic proteins such as the BH- only proteins such as Noxa[70] and APR-2[58]. Unlike various tumor types, particularly, those undergoing prolonged ER stress, the ER stress-dependent pathways such as IRE1α and ATF6 are persistent in melanoma cells[72]. Thus, it is expected that constitutive activation of both IRE1α and ATF6 would be associated with the development of melanoma resistance to anti-cancer agents[72]. Accordingly, the destruction of IRE1α and/or ATF6 signaling pathways has been reported to trigger apoptosis *via* mechanism mediated by PERK pathway[73]. The role of the PERK pathway in the modulation of ER stress-induced apoptosis has been demonstrated in various tumor types including melanoma *via* mechanism mediated by the BH3-only protein Bim[58,73].

Although the UPR is established as a cyto- protective response, excessive and/or persistent activation of ER stress-associated pathways can also trigger apoptosis[74]. However, the mechanism whereby UPR switches from the cyto- protection to apoptosis is thought to be the consequence of the attenuation of IRE1α and/or ATF6 activities[72,75]. The resistance of melanoma cells to most anti-cancer therapies during the course of anti-cancer-induced ER stress is attributed to the fact that the melanoma cells have adapted to ER stress. Although the molecular mechanisms that describe the contribution of ER stress in melanoma survival has been established, several studies revealed that the resistance of melanoma cells to ER stress-induced apoptosis results from the prolonged activation of the IRE1α and ATF6 pathways that, in turn, lead to the attenuation of the PERK signaling pathway[72]. Accordingly, the knockdown of IRE1α or ATF6 sensitizes melanoma cells to ER stress-induced apoptosis[33]. To that end, the destruction of the IRE1α/XBP-1 pathway along ER stress is expected to overcome melanoma resistance to ER stress inducers. The involvement of IRE1α in the activation of PI3K/Akt pathway together with the induction of Mcl-1 expression has been suggested to play an essential role in the modulation ER stress-induced survival of melanoma cells[76]. ATF6 is involved in the transcriptional regulation of both GRP78 and XBP-1 and thereby plays an important role in melanoma resistance to ER stress-induced apoptosis[77]. In conclusion, the differential response of various tumor types to PERK activation seems to rest on cellular factors and/or cell growth and survival pathways-dependent activation. Although the importance of IRE1/XBP-1 axis in tumor growth and survival has been established[78,79], its mechanistic role in the promotion of the XBP1 splicing processes and the subsequent effect on the components of the downstream signaling pathway have not been well characterized. More importantly, the activation of IRE1 kinase has been reported to be essential for the activation of c-Jun-N-terminal kinase, JNK and NF-κB pathways besides its role in the modulation of the induced unfolded protein response[79,80]. Upon the induction of ER stress, IRE1 kinase becomes capable of recruiting TRAF2. This results in the activation of both JNK and NF-κB pathways[81]. The mechanisms, involved in the modulation of ER stress are outlined in Figure 1.

ER stress-mediated pathways to autophagy in melanoma

Autophagy is a highly conserved degradation pathway that is responsible for the elimination of damaged cellular components. This process is implicated in several physiological and pathological processes leading to cell survival or cell death, and is characterized by the formation of double membrane autophagosomes[82]. The functional role of autophagy in melanoma has been reported in the context of *in vitro* analysis of chemotherapy-mediated effects in established melanoma cells[12,13]. We recently demonstrated that the induction of autophagic machinery in response to bortezomib protects melanoma cells from bortezomib-induced apoptosis[13]. Also, the induction of autophagy in response to the treatment with esomeprazole, a proton pump inhibitor, plays an essential role in the delay of melanoma cell death[32]. More importantly, the inhibition of ER stress-induced autophagy by the knockdown of Mcl-1, heat shock protein 70 (HSP70) or the inhibition of Bcl-2 potentiates bortezomib-induced apoptosis of melanoma cells[13,83]. Also, the combination of autophagy inhibitors 3-MA, bafilomycin A1 (BafA1) or LY294002 with the antiproliferative agents such as sanguilutine has the potential to reduce melanoma cell viability[84]. Orlotti *et al*[85] addressed the essential role of autophagy in the protection of melanoma cells from G-quadruplex ligand-induced-ER stress associated with DNA damage. Also, G-quadruplex ligand-induced autophagy has been suggested to be the consequence

of Ataxia Telangiectasia mutated-dependent DNA damage response as well as the transactivation of the cyclin-dependent kinase inhibitor 1A[84]. Although its mechanistic role in tumor survival and resistance to treatment with chemo-and radiotherapy has been established, autophagy can also enhance the killing efficiency of chemotherapy-based treatments in various tumor types including melanoma[86]. In recent years, autophagic cell death, also known as type Ⅱ apoptosis, gained more attention, as a potential therapeutic target for tumor treatment. Soares *et al*[87], demonstrated that the combination of Cl-IB-MECA inhibitor and paclitaxel can induce mTOR-dependent autophagic cell death, as well as caspase-dependent and/or independent apoptosis in melanoma cells. In addition, the potential of the micro-tubule poison, JG-03-14, to cause cytotoxic effects in melanoma cells both *in vitro* and *in vivo via* autophagy-dependent mechanism has been approved[88]. Thus, chemotherapeutic agents, whose cytotoxicity is mediated by autophagy-dependent mechanisms are considered to be suitable therapeutic approaches, particularly for tumors conferring resistance to anti-cancer agents-induced apoptosis. In addition, the identification of ER stress-associated pathways as a link between BRAF signaling and cytoprotective autophagy provides a potential therapeutic target for melanoma treatment[46]. Anti-cancer agents-induced autophagy is mostly resistant to several kinase inhibitors, particularly, those targeting the link between autophagic machinery and PI3K/AKT/mTOR pathway[89,90].

The common genetic alterations leading to the development of malignant melanoma are widely established to be the consequence of the activating mutations in *NRAS* and *BRAF* proto-oncogenes[91,92]. Also, genome-wide mutation detection in melanoma derived cell lines and primary tumors revealed significant alterations in the *BRAF* gene[93]. The most identified mutations were found to affect a single residue (V600E) that is located in the kinase activation domain of BRAF[94,95]. The importance of BRAF mutation is attributed to the potential role of RAF serine/threonine kinases, the most important key signaling components in the RAS pathways[96]. The clinical relevance of BRAF in melanoma is based on its mechanistic role in the activation of melanocytes in cAMP-dependent pathway in response to α-melanocyte-stimulating hormone-mediated activation of melanocortin receptor 1[97]. Accordingly, the mutation in the *BRAF* gene with its consequent impact on melanoma development and progression has gained increasing attention as a therapeutic target in melanoma. The development of a broad-spectrum of kinase inhibitors confirmed the clinical relevance of the inhibition of BRAF as an efficient therapeutic strategy for melanoma treatment. These kinase inhibitors have demonstrated the ability to inhibit BRAF, mutant BRAFV600E, and CRAF[98]. The most potent BRAF inhibitors, vemurafenib and dabrafenib, have demonstrated antitumor activity for advanced melanoma in phase Ⅲ trials, particularly in patients with BRAF mutations[99]. Also, MEK inhibitors, such as trametinib, showed significant antitumor activity in melanoma patients with a V600 BRAF mutation[100]. Other MEK inhibitors, such as Binimetinib, exhibit antitumor activity in patients with advanced melanoma, who demonstrate NRAS mutation[101]. Most importantly, the combination of BRAF inhibitors such as dabrafenib with MEK inhibitors such as trametinib have enhanced therapeutic benefits when compared with the response rate to dabrafenib alone[102]. Despite the demonstration of therapeutic progress by both BRAF and MEK inhibitors, most patients with metastatic melanoma fail to achieve a clinical cure[103]. The development of more effective therapeutics for advanced metastatic melanoma requires a direct evaluation of novel and innovative therapies. The roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and the implications for sensitivity to treatment of melanoma are outlined in Figure 2.

While MAP kinase pathways modulate autophagy-associated cell death[104], accumulated evidence demonstrates that autophagy also plays a role in the promotion of tumor resistance and survival *via* MAP kinase pathway-dependent mechanisms[13,105-107]. Specifically, the induction of cytoprotective autophagy counteracts MAP kinase-mediated pathways to apoptosis in response to chemotherapy-based treatments[13,108]. The presence of autophagosomes in tumor cells undergoing apoptosis in response to the treatment with chemotherapy is evidence for the ability of tumor cells to evade the cytotoxicity *via* autophagy-dependent pathways[109]. Thus, the inhibition of autophagic machinery induced by chemotherapeutics, such as bortezomib, may prove to be an effective therapeutic strategy[13]. In addition, these pathways may play a role in ER stress suppression of the anti-tumor efficiency of vemurafenib, dabrafenib and trametenib in melanoma patients harboring activating NRAS or BRAF mutations (Figure 2).

Anti-cancer agents affecting ER stress-associated pathways to apoptosis of melanoma

There are a number of United States Food and Drug Administration-approved anti-cancer agents that influence key components of ER stress-dependent pathways. For example, the ruthenium-derived compounds trigger the expression of ER stress proteins such as, Bip, XBP1, PDI, and CHOP leading to tumor growth inhibition or cell death[110,111]. Also, the anti-cancer agent 2-Hydroxyoleic acid triggers ER stress and autophagy in various human glioma cell lines[112]. Furthermore, the inhibition of the proteasome system with bortezomib overcomes resistance in a variety of tumors *via* mechanisms mediated by the accumulation of misfolded proteins that overwhelm the ER-associated degradation pathway that produce ER stress[113]. This mechanism is well described in multiple myeloma (MM) cells that constitutively express ER stress-associated survival factors that are essential for propagation and maintenance of MM cells[114,115]. Thus, proteasome inhibitors induce apoptosis in MM because the UPR is

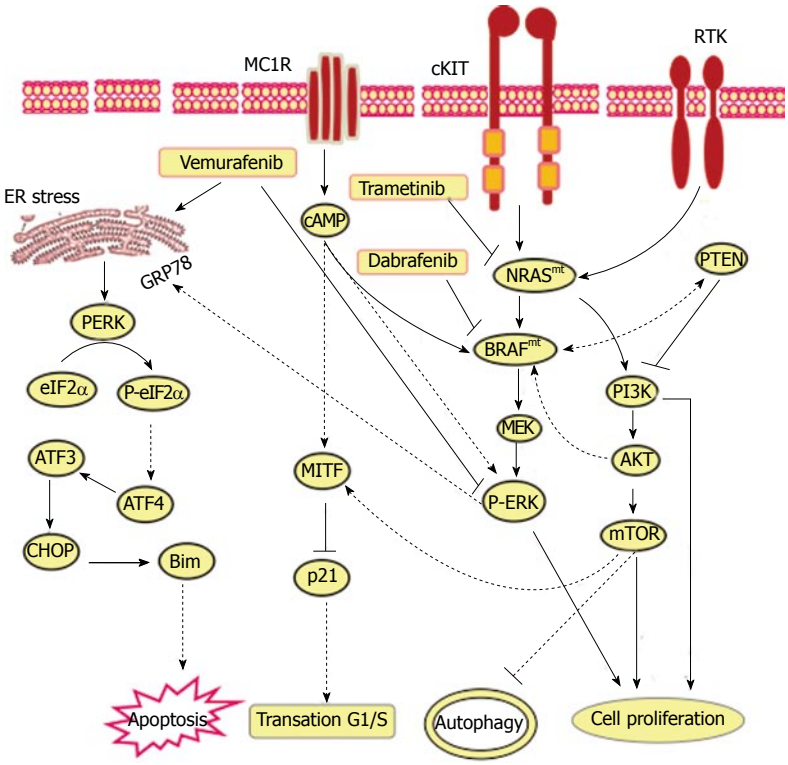

Figure 2 Proposed models for the mechanistic role of endoplasmic reticulum stress in the modulation of the anti-tumor efficiency of vemurafenib, dabrafenib and trametenib in melanoma patients harboring activating neuroblastoma RAS viral (v-ras) oncogene homolog or rapidly accelerated fibrosarcoma murine sarcoma viral (v-raf) oncogene homolog B mutations. The main function of MC1R, TRK on melanoma cell is to transmit extracellular signaling that is essential for the activation of RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways to mediate various cellular functions including melanoma initiation, progression and resistance. Thus, the exposure of melanoma ($BRAF^{mt}$ and $NRAS^{mt}$) cells to either Vemurafenib, Dabrafenib or Trametenib, respectively, results in inhibition of MC1R, cKIT and TRK-mediated activation of RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways. As consequence the inhibition of RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways results in the execution melanoma cell death *via* mechanism-mediated by ER stress-dependent pathways including PERK-eIF2α-ATF4-ATF3-CHOP-Bim pathway. NRAS: Neuroblastoma Rat Sarcoma viral (v-ras) oncogene homolog; BRAF: Rapidly Accelerated Fibrosarcoma murine sarcoma viral (v-raf) oncogene homolog B; MC1R: Melanocortin 1 receptor; TRK: CKIT and receptor tyrosine kinase; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT: Protein kinase B.

unable to mediate the degradation of the misfolded proteins[116]. In fact, compared to other cell lines, MM cells are the most sensitive to proteasome inhibitors-induced apoptosis *via* mechanism mediated by the activation of UPR-associated pathways including PERK and ATF4, and the pro-apoptotic target, CHOP[117].

The involvement of ER stress in the modulation of melanoma cell death in response to the treatment with anti-cancer agents has been studied extensively. For example, Syed *et al*[118], demonstrated that fisetin-induced apoptosis of melanoma cells is mediated by ER stress-associated pathways such as IRE1α, XBP1s, ATF4 and GRP78[119]. Also, the small molecule inhibitor honokiol, a potent anti-tumorigenic compound, has been shown to trigger apoptosis of melanoma cells *via* a mechanism mediated by the binding of honokiol to the unfolded ATPase domain of GRP78 leading to the induction of ER stress and pro-apoptotic associated pathways. Beck *et al*[120], addressed an important role for ER stress-associated pathways in the modulation of the anti-cancer agents. For example, in patients with BRAFV600E-mutated melanoma vemurafenib-induced apoptosis is associated with increased levels of the spliced isoform of the transcription factor, XBP1, a marker for the induction of ER stress, and with increased phosphorylation of the translation initiation factor eIF2α. Also, ER stressors such as diallyl trisulfide play a role in the sensitization of melanoma cells to death receptor- induced apoptosis[121]. Moreover, the role of ER stress-associated pathways in the modulation of the anti-tumor activity of the natural marine compound, 11-dehydrosinulariolide has been demonstrated[122]. Interestingly, the 11-dehydrosinulariolide compound was found to trigger apoptosis of melanoma cells *via* mechanism-mediated by both PERK/eIF2α/ATF4/CHOP and ATF6/CHOP pathways[122]. In another study, Hiscutt *et al*[123], demonstrated that knockdown of the X-linked inhibitor of apoptosis protein (XIAP) enhances both fenretinide and bortezomib-induced apoptosis of metastatic melanoma cells *via* ER stress-mediated pathways. Also, melanoma under ER stress shows more susceptibility to obatoclax-induced apoptosis[124]. Moreover, the role of ER stress-associated signaling pathways, GRP78, ATF6, IRE1α, and PERK/eIF2α has been reported to be essential for docatxel-induced apoptosis of melanoma[125]. More importantly, it has been suggested that the constitutively activated MEK/ERK pathway results in resistance of melanoma cells to ER stress-induced apoptosis. Accordingly, Jiang *et al*[48], demonstrated that the inhibition of MEK by U0126 inhibitor or by the knockdown of MEK1 by its specific siRNA sensitizes melanoma cells to tunicamycin- or thapsigargin-induced apoptosis. Also, the induction of ER stress by Tunicamycin can sensitize human melanoma cells to tumor necrosis factor-related apoptosis in response to ligand-induced apoptosis[126].

CONCLUSION

Although it has been demonstrated that ER stress-dependent pathways play a significant role in the regulation of tumor initiation and resistance, it is more difficult to confirm the hypothesis that ER is a valid therapeutic target for tumor treatment. The induction of UPR is a cellular mechanism that reduces or prevents the cytotoxic effect of anti-cancer treatment.

Accordingly, the destruction of key UPR components should provide an effective therapeutic strategy for melanoma treatment. Moreover, a functional analysis of UPR-mediated pathways, particularly those which are essential for cell survival or cell death, may help to identify key molecules of the aberrant pathways whose excessive activation and/or inhibition may overcome melanoma resistance to standard treatments. In addition, gaining an understanding of the molecular mechanisms of UPR may provide insight into the development of therapeutic strategies such as the development of small molecule inhibitors to control melanoma through the modulation of UPR signaling. Just as most current melanoma therapies were developed following a functional analysis of their ability to trigger mitochondrial dysregulation, ER stress-dependent pathways could provide new therapeutic targets designed to effect key components of aberrant signaling pathways.

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Enzymatic antioxidant system in vascular inflammation and coronary artery disease

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Abstract

In biological systems there is a balance between the production and neutralization of reactive oxygen species (ROS). This balance is maintained by the presence of natural antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. The enhancement of lipid peroxidation or the decrease of antioxidant protection present in metabolic diseases or bad lifestyle can induce endothelial dysfunction and atherosclerosis. Clinical studies have shown that oxidative stress can increase ROS reducing the formation of antioxidant defences, especially in subjects with coronary artery disease (CAD). Some observation indicated that in the early stages of the disease there is a homeostatic up-regulation of the antioxidant enzyme system in response to increased free radicals to prevent vascular damage. As soon as free radicals get to chronically elevated levels, this compensation ceases. Therefore, SOD and the other enzymes may represent a good therapeutic target against ROS, but they are not useful markers for the diagnosis of CAD. In conclusion antioxidant enzymes are reduced in presence of metabolic disease and CAD. However the existence of genes that promote their enzymatic activity could contribute to create new drugs for the treatment of damage caused by metabolic diseases or lifestyle that increases the plasma ROS levels.

Key words: Superoxide dismutase; Catalase; Glutathione peroxidase; Antioxidant enzyme; Coronary artery disease; Reactive oxygen species; Vascular inflammation

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Core tip: This review shows that antioxidant enzymes are very important factors for the prevention and treatment of atherosclerotic disease, but more studies are required to understand whether they can be used as markers for diagnosis of coronary artery disease. The presence of polymorphic genes that increases the activity and expression of these enzymes can be considered important for the development of new therapeutic strategies. In our opinion further efforts should be directed especially on this last point, in

order to find new therapies to increase the function of antioxidant enzymes in metabolic disease or other risk factors.

Lubrano V, Balzan S. Enzymatic antioxidant system in vascular inflammation and coronary artery disease. *World J Exp Med* 2015; 5(4): 218-224 Available from: URL: http://www.wjgnet.com/2220-315X/full/v5/i4/218.htm DOI: http://dx.doi.org/10.5493/wjem.v5.i4.218

INTRODUCTION

Oxygen has played a crucial role in the evolution[1-2] inducing the aerobic organisms to develop an adaptation to its toxicity by the presence of antioxidant systems. Oxygen is always metabolized to produce oxygen derived free radicals[3] (superoxide $O_2^{-\cdot}$, hydroxyl OH^-) and non-radical (hydrogen peroxide H_2O_2) all termed reactive oxygen species (ROS). In physiological condition there is a balance between the production and neutralization of ROS[4]. Small amount of ROS are constantly generated[5] and may often be useful for the immune system[6] and defense against microorganisms[5]. Conversely, high doses of ROS determine oxidative stress responsible for serious metabolic dysfunctions and damage to biological macromolecules[7]. The enhancement of lipid peroxidation or a decrease in antioxidant protection can frequently induce the reaction with the nucleophilic centers of the DNA, RNA and proteins leading to irreversible damage such as cytotoxicity, mutagenicity and carcinogenicity. For instance, intracellular $O_2^{-\cdot}$, hydroxyl radical (OH^-) and H_2O_2 play an important role in endothelial dysfunction, hypertension and atherosclerosis, inducing the expression of ICAM-1 and monocyte adhesion in endothelial cells[8,9]. To minimize the damage caused by free radicals the organism utilize enzymatic and non-enzymatic antioxidant systems. Of the first group are the superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione (GSH), while the second group consists of vitamin A, ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E)[10]. The fact that the activity of SOD is more intense in humans compared to other species could explain the longevity of our species suggesting that humans have better protection against ROS[11,12]. The imbalance between pro-oxidant and antioxidant systems can occur by an overproduction of ROS, (as the radical $O_2^{-\cdot}$, or OH^-), or because of the drastic reduction of antioxidant systems. Among the main sources of generation of ROS are the mitochondrial electron transport chain[13], the system of NAPDH oxidase and nitric oxide synthase (Figure 1). It has been known for years that NAD(P)H oxidase is a major source of superoxide in vascular tissue[14,15] and in cardiac cells[16].

$$NAD(P)H + 2O_2 \rightarrow NAD(P) + H^+ + 2O_2^{-\cdot}$$

And it has been demonstrated that its activity is increased by angiotensin II[17], thrombin, platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α) and lactosylceramide[18-20].

ENZYMATIC ANTIOXIDANT SYSTEM

SOD converts the highly reactive radical $O_2^{-\cdot}$ to the less reactive radical H_2O_2, which in turn can be destroyed by CAT or GPX, protecting the dehydratase (dehydratase hydroxyacid, aconitase, 6 phosphogluconate dehydratase, fumarase A and B). In humans, there are three forms of SOD: cytosolic (Cu, Zn-SOD), mitochondrial (Mn-SOD) and extracellular (EC-SOD)[21]. The respiratory chain in the mitochondria is the major source of oxygen radicals. Mn-SOD is of primary importance in removing $O_2^{-\cdot}$[22] and is essential for life. Cu, Zn-SOD seems to play an important role in the first line of antioxidant defense catalyzing the dismutation of $O_2^{-\cdot}$ radicals to form H_2O_2 and molecular oxygen, however, knock-outs experiments have shown that it is not essential for life[23]. EC-SOD is a tetrameric glycoprotein containing zinc and copper that has a high affinity for heparin. In mammalian tissues it is regulated by cytokines[24].

CAT consists of 4 ferriprotoporfirinici groups per molecule, is known as the most efficient enzyme since it is never saturated by the presence of H_2O_2[7]. CAT reacts with H_2O_2 and with proton donors (ROOH) producing H_2O. CAT protects the cells from the production of H_2O_2 playing an important role in the acquisition of tolerance to oxidative stress as an adaptive response of the cells[25].

GPX also catalyzes the reduction of a variety of hydroperoxides using GSH (ROOH and H_2O_2). The cells that contain low levels of GPX are much more susceptible to the toxicity of compounds such as adriamycin which produces hydroperoxides[26] and seems important as a line of defense against peroxynitrite.

ASSOCIATION BETWEEN ANTIOXIDANT ENZYME AND VASCULAR DISEASE

The increased oxidative stress is associated with the pathogenesis of coronary artery disease (CAD)[27,28]. Clinical studies have shown that oxidative stress can increase ROS reducing the formation of antioxidant defenses[27,28]. Some authors have demonstrated that the reduction of activity of antioxidant enzymes such as CAT, SOD and GPX facilitates the oxidative aggression to the cells, especially in subjects with CAD[29]. The study showed that in the early stages of CAD, SOD and CAT levels increased to protect and prevent lipid peroxidation whereas they decreased significantly with the worsening of the disease[29]. These observations indicate that in the early stages of the disease there is a homeostatic up-regulation involving the antioxidant

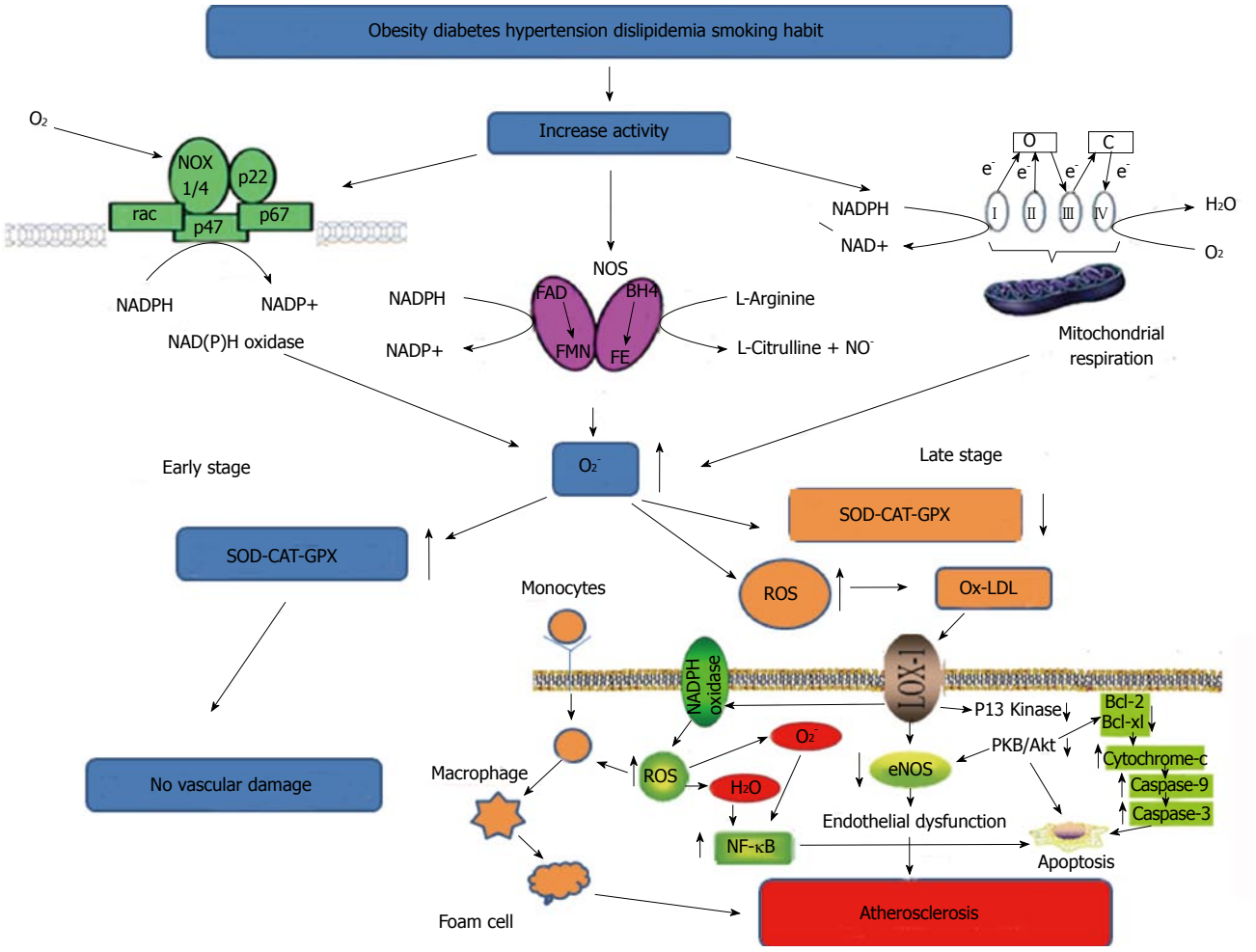

Figure 1 Biochemical events that favor the increase of reactive oxygen species. In the early stages of CAD, ROS do not cause damage due to the presence of an enzymatic compensatory mechanism. In late stage this mechanism is saturated and no longer allows an efficient defense, so that other biochemical events lead to vascular damage. ROS: Reactive oxygen species; SOD: Superoxide dismutase; CAT: Catalase; GPX: Glutathione peroxidase; CAD: Coronary artery disease; NF-κB: Nuclear factor-κB; NADPH: Nicotinamide adenine dinucleotide phosphate-oxidase; LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1; eNOS: Endothelial nitric oxide synthase; FAD: Flavin adenine dinucelotide; BH_4: Tetrahydrobiopterin; FE: Heme iron; FMN: Flavin mononucleotide.

enzyme system in response to increased free radicals to prevent vascular damage. In later stages of the disease, when free radicals get to chronically elevated levels, this mechanism, that has reached the saturation, suddenly crashes showing a reduction of antioxidant enzyme activities. In our study[30] in which we examined a population of CAD patients with different numbers of affected vessels, we did not find a significant difference in SOD levels even if higher values were observed in patients with three or four numbers of injured vessels (Table 1). We can speculate that in presence of high oxygen free radical levels, the compensatory response is only partially related to the activity or expression of the EC-SOD enzyme linked to the damaged artery. However, our data do not disagree with the data of Gupta *et al*[29] concerning the patients in the late stages of the disease, when the vascular damage is already present; in fact they did not find any difference in SOD and CAT activity. The difference is mainly in the fact that the authors have also examined patients in the early stages of CAD, when the antioxidant enzyme system was not yet saturated. Therefore, EC-SOD do not appear a useful marker for the diagnosis of CAD or to stratify the patients population[30] (Table 1), but it may represent a good therapeutic target against ROS in CAD. In another study, Kotur-Stevuljevic *et al*[31] found that the enzymatic activity of erythrocyte SOD in patients with CAD was significantly decreased comparing to healthy volunteers. A significant difference was also evident among patients with stenosis less than 50% compared to those with stenosis higher than 50%. However, the activity of SOD in patients with stenosis less than 50% was not significantly different from the control group, showing that also a compensatory mechanism did not exist in this population. This means that the homeostatic enzyme response occurs when the vascular damage has not yet been produced. This phase could represent an important time point for therapeutic treatments that stimulate the enzyme system for a longer time or with natural scavenger to massively reduce the presence of ROS produced by metabolic diseases or from unhealthy lifestyle. From the clinical point of view

Table 1 Inflammatory parameters, and serum levels of extracellular superoxide dismutase and free radicals in a group of patients with coronary artery disease and healthy volunteers drawn from the study of Lubrano *et al*[30]

Number of injured vassels	IL-6 (pg/mL)	TNF-α (pg/mL)	CRP (mg/dL)	Peroxy radicals (UC)	EC-SOD (U/mL)
Controls	1.05 ± 0.2	2.5 ± 1.1	0.15 ± 0.03	197 ± 15.5	2.91 ± 0.4
1	2.7 ± 0.7	1.05 ± 0.3	0.83 ± 0.7	241 ± 30.7	2.9 ± 0.4
2	2.6 ± 1.7	1.34 ± 0.2	1.6 ± 0.1	246 ± 12.5	2.7 ± 0.6
3	3.3 ± 1[a]	0.98 ± 0.4	1.1 ± 0.7	272 ± 20.2	3.8 ± 0.7
4	3.5 ± 0.6[a]	3.9 ± 1.5	1.4 ± 1.1	273 ± 30.5	5.1 ± 1.3

[a]$P < 0.05$. TNF-α: Tumor necrosis factor-α; EC-SOD: Extracellular superoxide dismutase; CAD: Coronary artery disease; CRP: C-reactive protein.

it would be important to combine the analysis of SOD CAT, GPX with markers of vascular inflammation such as cytokines and C-reactive protein in cases where it is already established the presence of risk factors such as hypertension, diabetes or hypercholesterolemia, but in the absence of plaque formation. The intimal thickening, effect induced by endothelial activation, is an early event to consider. At this stage the cells begin to produce inflammatory cytokines that attract monocytes, adhesion molecules, receptors for oxidized lipoproteins and a massive increase of ROS. Of course, more studies are needed to determine the precise role of these enzymes in protecting the arteries from ROS damage, in order to clarify whether they can be inserted in both the prevention and the treatment of atherosclerotic disease.

INFLUENCE OF THE MAIN RISK FACTORS IN THE MODULATION OF ANTIOXIDANT ENZYME

Obesity has been considered an important factor in causing various health problems, especially in vascular disease[32]. It has been observed that the adipose tissue secretes adipokines, responsible for the production of ROS, and independent factors, for the generation of systemic oxidative stress[33]. The persistence of obesity implies an increase of inflammatory cytokines and an excessive consumption of oxygen, which generates free radicals in the respiratory chain coupled to oxidative phosphorylation in mitochondria. In the long term, the accumulation of fat may deplete the sources of antioxidants and significantly decrease the activity of enzymes such as SOD, CAT and GPX and the presence of non-enzymatic factors such as vitamin E, vitamin C and β-carotene[33].

Even in the case of diabetes there is convincing experimental evidence and clinical trials that have demonstrated that the onset of the disease is closely associated with oxidative stress[34,35]. Potential sources of ROS in diabetes can be justified by the increase in glucose oxidation and by the changes of redox balance through a cascade of ROS generated by mitochondria. This process has been associated with the onset of type 1 diabetes (DM1) caused by pancreatic beta cell apoptosis, and the onset of type 2 diabetes (DM2) caused by insulin resistance[36]. Some authors demonstrated that high glucose levels could stimulate cytochrome P450 activity by excessive nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) produced by glucose metabolism[37]. In addition, ketosis, a hallmark of DM1, seems to increase the production of oxygen radicals in this patients[38]. The reduced enzymatic activity of CAT, SOD, GSH-PX, and glutathione reductase (GSH-Rx), as well as high levels of thiobarbituric acid (TBARS), an indirect measure of the production of ROS, that seem to be consistently high in diabetes[39] are important indices for interpreting the extent of the disease. Recent studies have shown that the levels of SOD and glutathione S-transferase activities were significantly lower in patients with T2DM compared to healthy subjects[40]. It is known that the use of vitamin E as a dietary supplement for patients with CAD entails a significant benefit in reducing the symptoms of angina pectoris[41]. In diabetic rats the beneficial effect of vitamin E showed the delay of onset of coronary atherosclerosis compared to untreated. The slowing of the development of the disease was due to a reduction in oxidative stress, and not secondary to a decrease in the glucose or cholesterol in plasma, for the fact that the respective plasma concentrations remained unchanged in the diabetic mice supplemented with vitamin E[42]. Other studies have supported these results, in fact, it was observed that a triple antioxidant therapy (Vitamin E, lipoic acid, and vitamin C) in diabetic volunteers attenuated oxidative stress reducing the formation of methemoglobin *in vitro* and in glycated hemoglobin *in vivo*[43]. Numerous clinical studies have shown a decrease in EC-SOD in African Americans with hypertension, in patients with vasospastic angina, calcific aortic stenosis and in patients with DM2, compared with control subjects[44,45]. Furthermore, it was observed that the standard dietary treatment for type 2 diabetic patients produces an increase of the SOD and GPX activity[46].

It is evident that the mechanism, that renders low-density lipoprotein a good substrate for the production of foam cells and the initiation of atherosclerotic events, is their oxidative modification[28]. As for other conditions, some investigators have shown that the overexpression of antioxidant enzymes can slow the progression of atherosclerosis[47]. Tests carried out on animals have found that the use of natural antioxidants supplements leads to the increase of enzyme activities[48], whereas

the intake of excessive dietary lipids and therefore an excess of energy and cholesterol has a negative influence on antioxidant enzymes. A negative correlation between dietary cholesterol and the markers of antioxidant enzyme activity, including CAT and GPX, was observed. The authors have shown a significant improvement in erythrocyte antioxidant capacity, as increased activity for SOD, CAT and GPX in children with hypercholesterolemia who have followed a diet with reduced saturated fat and introduction of several fatty acids for 6 mo[49].

CLINICAL IMPLICATIONS

Increased expression of CuZn-SOD (SOD1) protects muscle cells from oxidative damage. It was observed that overexpression of *SOD1* gene inhibits the DNA binding activity of activator protein-1 and NF-κB. Interesting prospects are given by the fact that the substitution of valine with alanine has been shown to induce an increase of 30%-40% in the activity Mn-SOD in the mitochondria with consequent reduction of the risk of CAD and acute myocardial infarction[50]. Even the overexpression of GPX reduces oxidation of the phospholipids, the formation of hydroperoxides of cholesterol, as well as pro-inflammatory lipid peroxides generated by LPO and COX, reducing oxidative stress and vascular atherosclerosis progression. From these observations we conclude that the antioxidant enzyme system is inversely associated with a high-fat diet, and as previously described, the increase in vitamin E, vitamin C, and β-carotene is associated with the strengthening of SOD, therefore the feeding is an important factor in the prevention and treatment of oxidative damage caused by ROS. In the near future it will be possible to study also the genetic polymorphism. The existence of a gene that promotes the enzymatic activity of SOD can contribute to create new drugs for the prevention of damage caused by metabolic diseases or lifestyle that increases the plasma levels of ROS. We believe that further studies should be performed to determine if there is a mechanism of compensation of the antioxidant enzyme system induced by the presence of ROS, and in this case to understand when it begins and what is its intensity. This fact is not very clear from previous studies, because if on one hand it seems to develop before vascular lesion, one the other hand it has never been observed in the presence of metabolic diseases, when the vascular damage has not yet happened.

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MINIREVIEWS

Disease control by regulation of P-glycoprotein on lymphocytes in patients with rheumatoid arthritis

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Abstract

The main purpose of treatment of rheumatoid arthritis (RA) with disease modifying antirheumatic drugs (DMARDs) is to control activation of lymphocytes, although some patients do not respond adequately to such treatment. Among various mechanisms of multidrug resistance, P-glycoprotein (P-gp), a member of ATP-binding cassette transporters, causes drug-resistance by efflux of intracellular drugs. Certain stimuli, such as tumor necrosis factor-α, activate lymphocytes and induce P-gp expression on lymphocytes, as evident in active RA. Studies from our laboratories showed spontaneous nuclear accumulation of human Y-box-binding protein-1, a multidrug resistance 1 transcription factor, in unstimulated lymphocytes, and surface overexpression of P-gp on peripheral lymphocytes of RA patients with high disease activity. The significant correlation between P-gp expression level and RA disease activity is associated with active efflux of drugs from the lymphocyte cytoplasm and in drug-resistance. However, the use of biological agents that reduce P-gp expression as well as P-gp antagonists (*e.g.*, cyclosporine) can successfully reduce the efflux of corticosteroids from lymphocytes *in vitro*, suggesting that both types of drugs can be used to overcome drug-resistance and improve clinical outcome. We conclude that lymphocytes activated by various stimuli in RA patients with highly active disease acquire P-gp-mediated multidrug resistance against corticosteroids and probably some DMARDs, which are substrates of P-gp. Inhibition/reduction of P-gp could overcome such drug resistance. Expression of P-gp on lymphocytes is a promising marker of drug resistance and a suitable therapeutic target to prevent drug resistance in patients with active RA.

Key words: *Multidrug resistance 1* gene; P-glycoprotein; Lymphocytes; Disease activity; Rheumatoid arthritis

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Core tip: In patients with refractory rheumatoid arthritis (RA) and high disease activity, overexpression of P-glycoprotein (P-gp) on lymphocytes can cause resistance to anti-rheumatic drugs through efflux of intracellular drugs from these cells. Lymphocytes activated by various stimuli, including tumor necrosis factor-α in RA patients apparently acquire P-gp-mediated multidrug resistance against certain anti-rheumatic drugs, which are substrates of P-gp. The use of biological agents that reduce P-gp expression as well as P-gp antagonists can successfully reduce the efflux of drugs from lymphocytes, suggesting that they can be used to overcome drug-resistance and improve clinical outcome.

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INTRODUCTION

Rheumatoid arthritis (RA) is manifested by inflammatory and chronic destruction of multiple joints with occasional systemic organ complications based on immune abnormality[1]. Poor control of RA is associated with severe painful disability and impairments at work and life. The strategic treatment to control immune-mediated synovial inflammation, joint destruction and extra-organ manifestation is by early intervention with synthetic or biological disease modifying anti-rheumatic drugs (DMARDs). Early treatment of RA with DMARDs can result in prevention of joint destruction and a better long-term outcome[2]. DMARDs commonly target lymphocytes and the cytokines produced by these cells, which play an important role in the pathogenesis of RA[3]. However, we often encounter RA patients who are refractory to these DMARDs and fail in the control of high disease activity[4]. Thus, overcoming activated lymphocytes involved in drug-resistance is an important goal of the treatment in some refractory RA patients. P-glycoprotein (P-gp) is a member of ATP-binding cassette transporters and is induced on the cell membrane by certain stimuli. P-gp transports multiple drugs from the cytoplasm to the cell exterior, resulting in the development of drug resistance. Here, we discuss the importance of P-gp on activated lymphocytes and its relevance to multidrug-resistance and the potential for treatments targeting P-gp on lymphocytes to overcome drug-resistance in refractory patients with RA.

MECHANISMS OF DRUG RESISTANCE MEDIATED BY P-GP

P-gp is encoded by the multidrug resistance-1 (MDR-1)[5-7], a member of the ATP-binding cassette transporter superfamily of genes. P-gp is recognized by structurally diverse, hydrophobic/amphiphilic substrates, ranging from 300 to 2000 Da, catches these substrates like a "vacuum cleaner" during passing through the cell membrane, and pumps them out of the cells in a manner dependent on the energy of ATP hydrolysis. Therefore, Corticosteroids, certain immunosuppressants and DMARDs, including antimalarial drugs, are extruded from lymphocytes with overexpression of P-gp, which leads to reductions in the concentrations of these drugs in cytoplasm and failure of their intracellular effects (Table 1)[8-13]. Indeed, P-gp-mediated efflux of corticosteroids from lymphocytes can result in low cytoplasmic corticosteroid concentrations and development of corticosteroid resistance in systemic lupus erythematosus[14]. Thus, excessive excretion of the drugs from P-gp-overexpressing lymphocytes can be involved in the drug-resistance often observed in patients with RA.

Table 1 Relation of P-glycoprotein with disease modifying antirheumatic drugs and immunosuppressants

Drug	Pharmacological substrates of P-glycoprotein	Competitive inhibitor of P-glycoprotein
Corticosteroids	Yes	No
Cyclosporine	Yes	Yes
Tacrolimus	Yes	Yes
Methotrexate	No	No
Leflunomide	No	No
Hydroxycloroquine	Yes	Yes
Sulfasalazine	Yes	Unknown
D-penicillamine	Yes	Unknown
Colchicine	Yes	No
Cyclophosphamide	No	No
Azathioprine	No	No

THE REGULATION OF P-GP EXPRESSION ON LYMPHOCYTES

Endothelial cells of the blood brain barrier and various epithelial cells show congenial expression of P-gp for protection of cells from toxic substances. In contrast, P-gp expression on normal resting lymphocytes is marginal but can be induced by certain stimuli[15-17]. Overexpression of P-gp appears to be closely associated with the nuclear localization of human Y-box-binding protein-1 (YB-1) in various tumors[18]. YB-1, which is a member of the DNA/RNA-binding protein family containing a cold-shock domain, is activated in response to various genotoxic stimuli and drives the transcription of *MDR-1* gene[18]. We have demonstrated that lymphocytes can be activated by various stimuli, such as cytokines and extracellular matrix to induce P-gp expression on lymphocytes, based on the following sequence of events; activation and translocation of YB-1 by IL-2, tumor necrosis factor-α (TNF-α) (Figure 1A) and fragmented hyaluronan, transcriptional activation

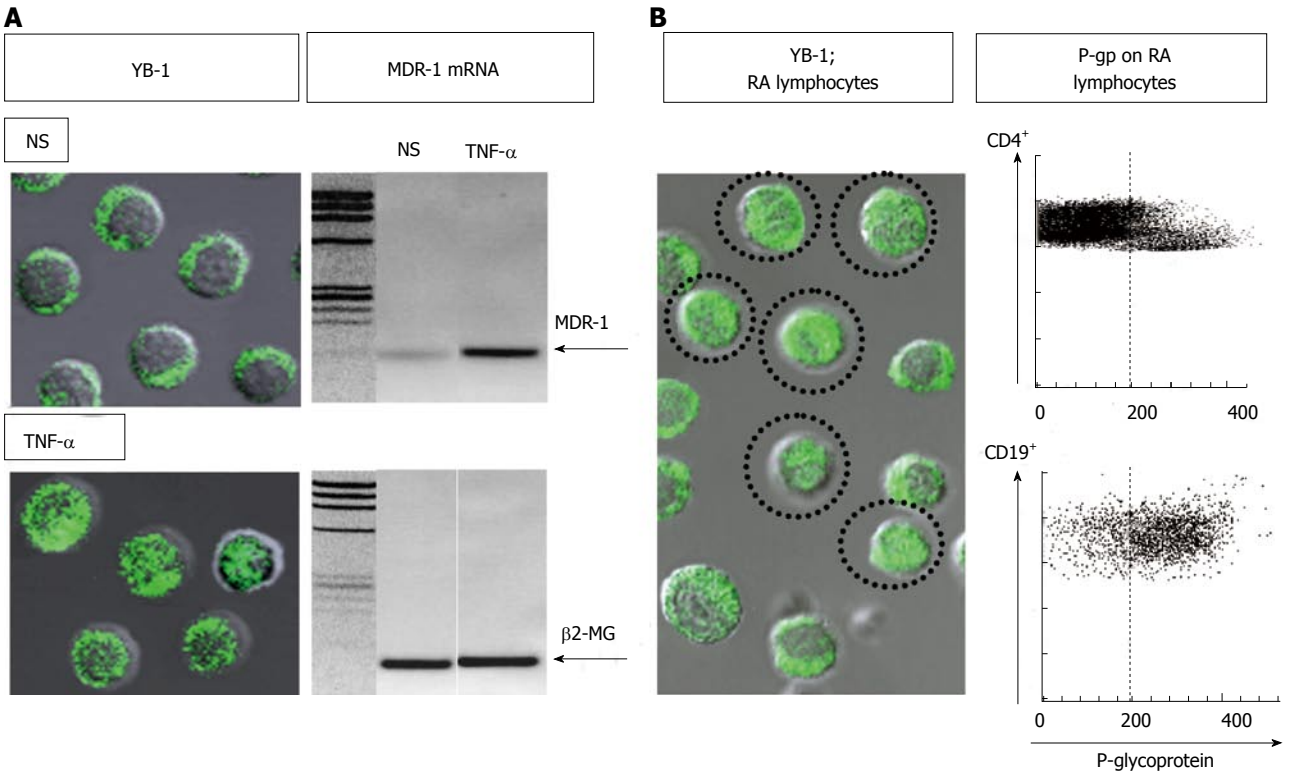

Figure 1 Up-regulation of nuclear translocation of Y-box-binding protein-1, transcription of multidrug resistance 1 in lymphocytes, and P-glycoprotein expression on lymphocytes. A: Left: Immunostaining and confocal microscopy analysis of Y-box-binding protein-1 (YB-1) in 1 × 10^5 of peripheral blood mononuclear cells (PBMCs). YB-1 was expressed in the cytoplasm of all non-stimulated PBMCs (NS). In contrast, nuclear translocation of YB-1 was induced in 30% or more of PBMCs incubated with 10 ng/mL of tumor necrosis factor-α (TNF-α). Immunostaining for YB-1 using a specific antibody (Ab) against YB-1[19] with FITC-conjugated anti-rabbit IgG Ab (BD Biosciences Pharmingen). Confocal analysis of YB-1 using a LSM 5 Pascal invert Laser Scan Microscope (Carl Zeiss Microscope Systems, Germany). Magnification, × 600; Right: Multidrug resistance-1 (MDR-1) mRNA expression was examined by RT-PCR using total RNA extracted from PBMCs incubated with 10 ng/mL of TNF-α or no stimulation (NS). The primer sequences were as follows: human β2-microglobulin forward 5'-ACCCCCACTGAAAAAGATGA-3', reverse 5'-ATCTTCAAACCTCCATGATG-3'; human MDR-1 forward 5'-CCCATCATTGCAATAGCAGG-3', reverse 5'-GTTCAAACTTCTGCTCCTGA-3'. Amplified products were electrophoresed with Marker 4 (Nippon Gene, Tokyo) on 3% agarose gels; B: Spontaneous nuclear translocation of YB-1 and P-glycoprotein (P-gp) expression on lymphocytes from a typical patient with active rheumatoid arthritis (RA). Left: Immunostaining and confocal microscopy analysis of YB-1 in 1 × 10^5 of PBMCs. YB-1 was expressed in the nuclei of a proportion of unstimulated PBMCs (encircled cells). Magnification, × 600; Right: P-gp expression on $CD4^+$ and $CD19^+$ peripheral blood lymphocytes. The dotted line represents the gate set to discriminate negative from positive stained cells as determined by control FITC-conjugated anti-mouse IgG Ab. The specific antibodies for staining and flow cytometric analysis were as follows: staining for P-gp using MRK16 (a specific monoclonal Ab against P-gp; Kyowa Medex, Tokyo) with FITC-conjugated goat anti-mouse IgG Ab (BD Biosciences Pharmingen), cy-chrome-conjugated CD4 monoclonal Ab, cy-chrome-conjugated CD19 monoclonal Ab (BD Biosciences Pharmingen).

of MDR-1 by activated YB-1, P-gp expression on the cell surface membrane of lymphocytes, expelling added dexamethasone from lymphocytes, leading to a fall in intracellular dexamethasone concentration[16,17]. Serum and synovial concentrations of IL-2 are high in patients with active RA[19,20]. TNF-α is a clinically validated pathogenic factor in inflammatory erosive arthritis in RA and is pivotal target for directed biologic intervention[3,21-23]. Fragmented hyaluronan is increased in the RA synovium and synovial fluid[24,25]. The enhanced production of fragmented hyaluronan is due to increased digestion of native hyaluronan, which is increased in inflammatory foci like synovitis by inflammatory cytokines including IL-1β and TNF-α[26] and by oxygen-derived free radicals[24,25]. Overexpression of P-gp on lymphocytes induced by these stimuli[15,16], which also play important roles in the disease activity of RA, parallels the activation of lymphocytes. P-gp expression is preferentially high on CD69-expressing lymphocytes, which is a well-defined marker of early activation of lymphocytes[16,17]. Actually, lymphocytes in highly active RA patients, *i.e.*, pathologically active lymphocytes, show accumulation of YB-1 in the nuclei and overexpression of P-gp on the cell surface membrane (Figure 1B). Thus, lymphocyte activation by certain stimuli induces YB-1 activation followed by P-gp overexpression, resulting in acquisition of multidrug resistance mediated by P-gp (Figure 2A and B, and Table 1). Therefore, the presence of active lymphocytes that have acquired P-gp-mediated multidrug resistance are probably involved in the failure of disease control in patients with active RA.

CLINICAL VALIDATION OF THE RELATIONSHIP BETWEEN P-GP-EXPRESSING RA LYMPHOCYTES AND DRUG RESISTANCE

The expression level of P-gp is significantly high on most peripheral $CD4^+$ T cells and $CD19^+$ B cells in RA

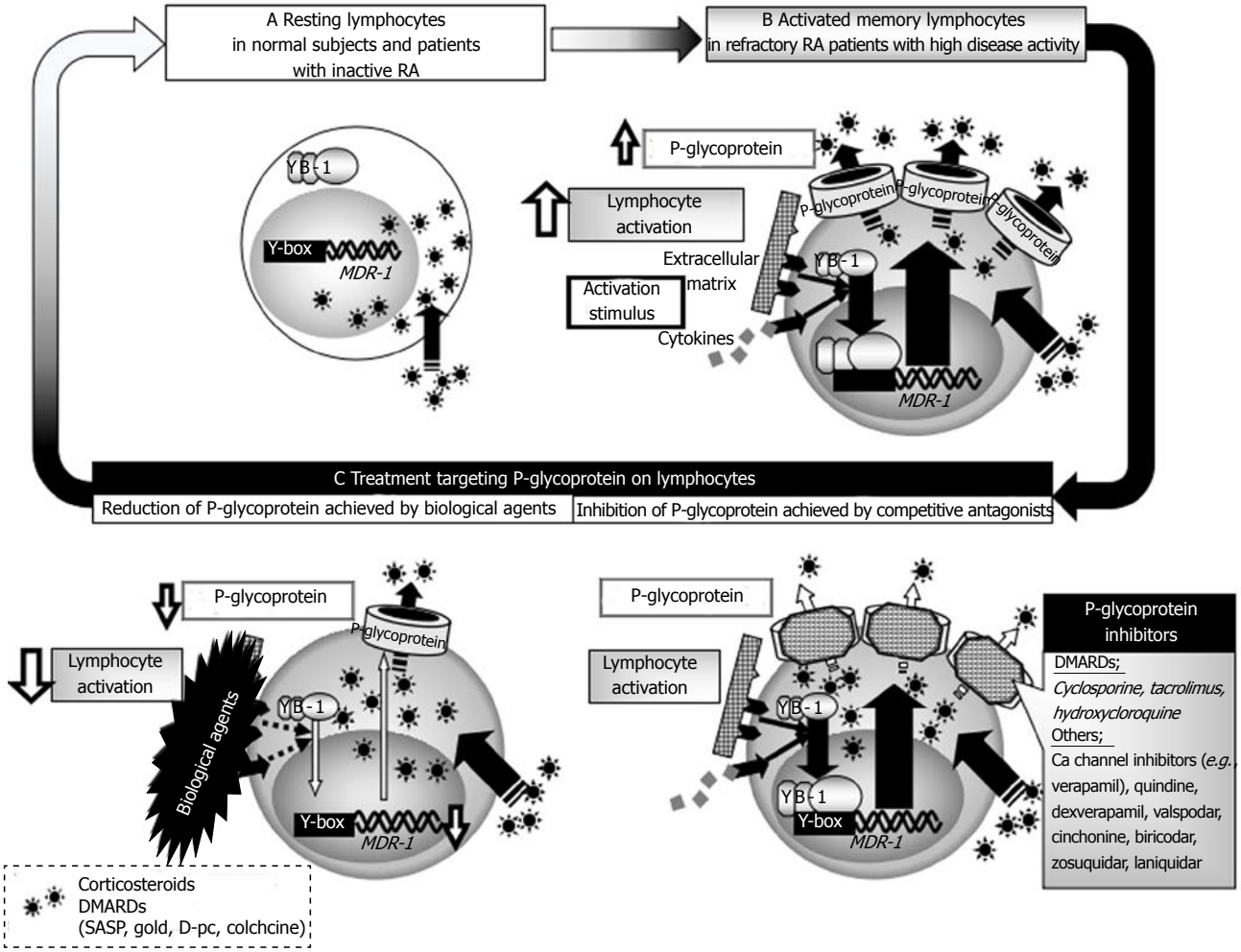

Figure 2 Schematic diagram of the relevance of P-glycoprotein to drug resistance in rheumatoid arthritis. A: Y-box-binding protein-1 is located in the cytoplasm of lymphocytes and P-glycoprotein (P-gp) is only marginally expressed on normal lymphocytes of normal subjects and patients with inactive RA; B: In patients with highly active rheumatoid arthritis (RA), various stimuli induce P-gp expression on lymphocytes, which leads to active efflux of drugs from lymphocytes, resulting in drug-unresponsiveness and failure to control disease activity; C: Reduction of P-gp achieved by intensive immunosuppressive therapy and inhibition of P-gp by competitive antagonists, such as cyclosporine, could overcome P-gp-related drug-resistance in patients with highly active RA. DMARDs: Disease modifying antirheumatic drugs; MDR-1: Multidrug resistance 1.

patients, but marginal in normal subjects. Evidence indicates that P-gp expression level on lymphocytes characteristically correlates significantly with RA disease activity, estimated by the disease activity score (DAS) 28-3[27], and that its levels on $CD4^+$ T cells and $CD19^+$ B cells are markedly increased in patients with inadequate response to one DMARD treatment with corticosteroids or to at least two DMARDs for at least 2 years, compared with responders and normal volunteers (Figure 3).

To elucidate the relationship between P-gp expression and P-gp-mediated efflux of intracellular drugs *in vitro*, intracellular and extracellular concentrations of dexamethasone, a representative substrate of P-gp (Table 1), were determined by the C/M ratio, an index of intracellular concentration of dexamethasone (C) and extracellular concentration of dexamethasone in the conditioned medium (M). Using this method, we analyzed *in vitro* peripheral blood lymphocytes from RA patients with highly active disease (DAS 28-3 > 5.1). Significantly low intracellular dexamethasone levels were found in P-gp-overexpressing lymphocytes, compared with those from RA patients with mild to moderate active disease (DAS 28-3 < 5.1) and normal volunteers. The above findings indicate that overexpression of P-gp on activated lymphocytes leads to active efflux of certain intracellular drugs, substrates of P-gp, from the cells, and results in the development of drug resistance and failure of the disease control in highly active RA (Figure 2B). Taken together, it is possible that treatment that targets P-gp on lymphocytes could overcome drug-resistance in refractory patients with RA. Treatment modalities that target P-gp are classified into those that reduce P-gp expression and others that inhibit P-gp function.

BIOLOGICAL AGENTS CAN OVERCOME P-GP-MEDIATED DRUG RESISTANCE IN PATIENTS WITH REFRACTORY RHEUMATOID ARTHRITIS

TNF-α is a central player in the inflammatory process

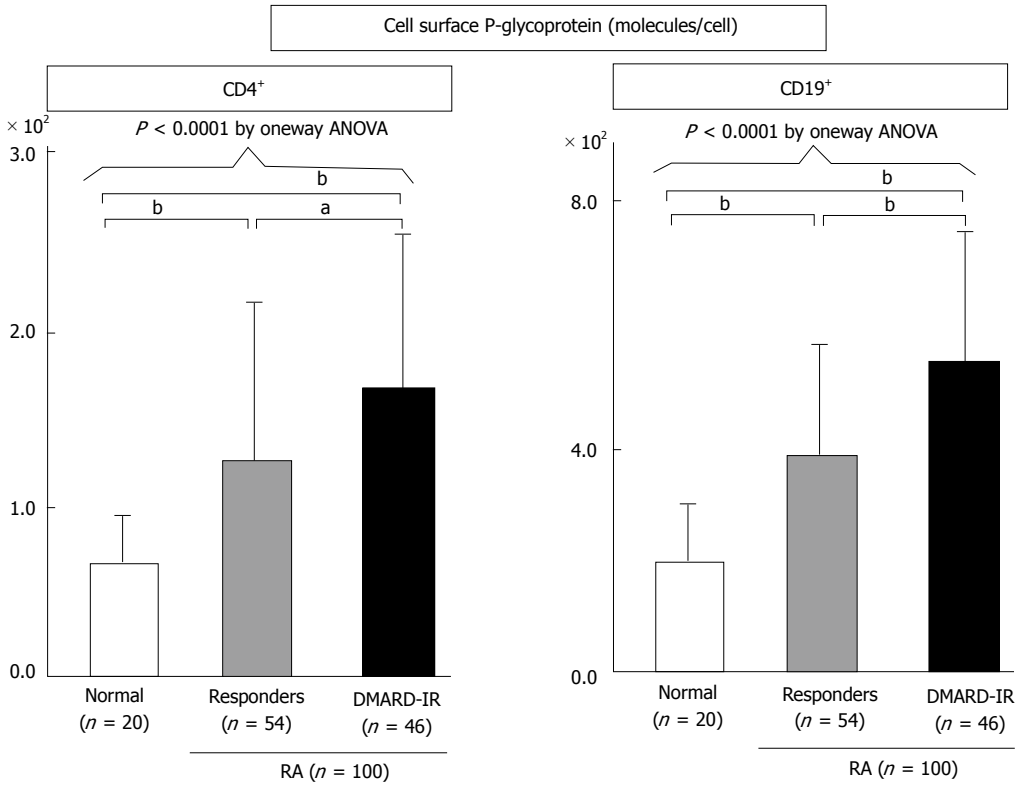

Figure 3 Expression of P-glycoprotein on lymphocytes from patients with refractory rheumatoid arthritis, as determined by flow cytometry. P-glycoprotein (P-gp) expression on $CD4^+$ or $CD19^+$ peripheral blood lymphocytes from 20 normal volunteers (open bar) and 100 RA patients [responders, hatched bars; with inadequate response to DMARDs (DMARD-IR), closed bars]. The specific antibodies for staining and flow cytometric analysis were as follows: staining for P-gp using MRK16 [a specific monocloncal antibody (Ab) against P-gp; Kyowa Medex, Tokyo] with FITC-conjugated goat anti-mouse IgG Ab (BD Biosciences Pharmingen), cy-chrome-conjugated CD4 monoclonal Ab, cy-chrome-conjugated CD19 monoclonal Ab (BD Biosciences Pharmingen). Data represent the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Values are mean ± SD of independent experiments. One-way ANOVA were used to compare data between groups. $^aP < 0.05$, $^bP < 0.01$, by multiple comparison. DMARD: Disease modifying antirheumatic drug; RA: Rheumatoid arthritis.

of RA, as it exacerbates erosive synovitis and enhances disease activity, and is thus an excellent molecular target for directed biologic intervention[3,21-23]. Infliximab and etanercept, two highly effective antagonists of TNF-α, have revolutionized treatment strategies for RA. In one study, infliximab improved clinical disease activity in intractable RA through significant reduction of P-gp expression levels on $CD4^+$ T cells and $CD19^+$ B cells, which were otherwise uncontrolled by treatment with MTX[27]. In another study published by our group, etanercept significantly reduced CD69 and P-gp expression on $CD4^+$ T cells and $CD19^+$ B cells in each of 11 patients with intractable RA, including two who experienced secondary loss of infliximab efficacy and eight who did not use MTX[17]. The above two studies demonstrated that treatment with TNF-α antagonists can improve RA disease activity within two weeks[17,27], and allow tapering or withdrawal of steroid therapy[17]. These effects were associated with recovery of intracellular dexamethasone levels in lymphocytes accompanied by falls in P-gp levels in these cells[17,27]. The results imply that intensive treatment with TNF-α antagonists reduces P-gp expression and results in rescue of DMARD and steroid concentrations in the cytoplasm of lymphocytes.

TNF antagonists act extracellularly and inhibit lymphocyte activation without being affected by P-gp[17]. Inhibition of lymphocyte activation by TNF-α antagonists probably suppresses YB-1-driven transcriptional activation of MDR-1 and P-gp expression on lymphocytes, and excretion of multiple drugs from the cytoplasm to the extracellular compartment. Therefore, inhibition of lymphocyte activation by TNF-α antagonists can probably thwart P-gp-mediated treatment resistance in refractory patients with RA (Figure 2C).

Translated clinically, when treatment with DMARDs and corticosteroids fails to control high disease activity in RA patients, administration of DMARDs or immunosuppressants that escape from P-gp-orchestrated excretion (Table 1), is a better treatment option. Furthermore, treatment with biological agents including TNF-α antagonists, should be initiated.

P-GP COMPETITORS REGULATE P-GP-MEDIATED DRUG RESISTANCE IN PATIENTS WITH REFRACTORY RHEUMATOID ARTHRITIS

The immunosuppressants cyclosporine and tacrolimus act as calcineurin inhibitors and inhibit cytokine production promoted by NF-AT, as well as act as P-gp-

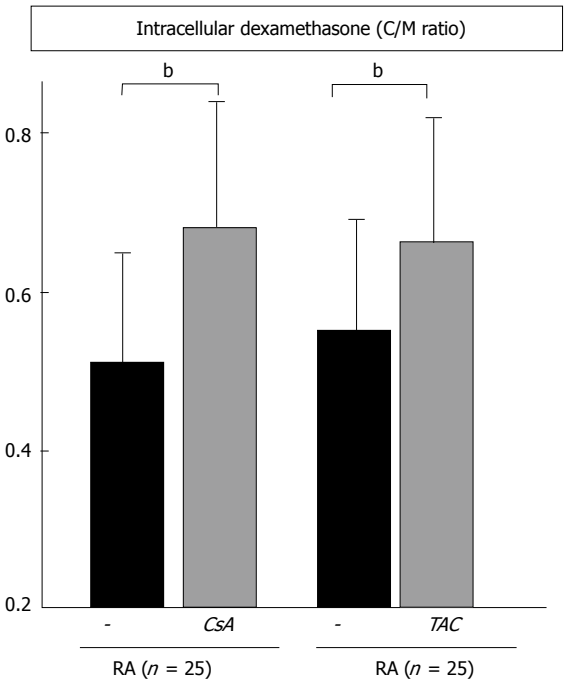

Figure 4 Inhibition of P-glycoprotein-related dexamethasone efflux by cyclosporine and tacrolimus. The C/M ratio in peripheral blood mononuclear cells of 25 rheumatoid arthritis (RA) patients was measured in the absence or presence of 100 ng/mL of cyclosporine (CsA), or 10 ng/mL of tacrolimus (TAC). Data are mean ± SD. $^{b}P < 0.01$, by the paired *t* test.

competitive inhibitors. Recent therapeutic intervention studies have investigated the efficacy of P-gp competitors, such as cyclosporine and its derivatives, in overcoming P-gp-mediated treatment resistance[28,29]. We have demonstrated that two P-gp competitive immunosuppressants, cyclosporine and tacrolimus, inhibited dexamethasone excretion from IL-2-activated lymphocytes, and that this action was concentration-dependent. In addition, treatment with these competitors resulted in recovery of intracellular dexamethasone concentrations in IL-2-activated lymphocytes by doses lower than trough levels measured clinically when these agents are used as calcineurin inhibitors[15]. Actually, the addition of low doses of tacrolimus and cyclosporine to lymphocytes harvested from highly active RA patients, resulted in recovery of intra-lymphocytes dexamethasone concentrations (Figure 4).

In our clinical trial, we experienced refractory patients with high disease activity who were treated with low-dose cyclosporine or tacrolimus and showed significant improvement of clinical features within two weeks, which was not accompanied by falls in P-gp expression levels on lymphocytes[30]. One representative case was a 56-year-old woman with P-gp-overexpressing lymphocytes. Despite treatment with MTX, sulphasalazine, and D-penicillamine (D-pc), the RA disease activity flared several times and the joints destruction rapidly progressed during a period of two years. Low-dose cyclosporine therapy (serum cyclosporine concentration < 100 ng/mL), added to MTX with D-pc, markedly improved RA disease activity and normalized CRP and MMP-3 within two months. This outcome suggests that cyclosporine and tacrolimus, administered at low doses and over a relatively short period of time than necessary for NFAT inhibition, competitively inhibit elimination of intracellular drugs through P-gp on lymphocytes without simultaneously reducing P-gp expression, and can thus be used to overcome drug resistance and result in improvement of clinical features. We propose that competitors of P-gp, including cyclosporine, tacrolimus and hydroxy-cloroquine (Table 1), are potentially effective therapies for RA patients with high disease activity who are refractory to treatments with conventional DMARDs (Figure 2C).

CONCLUSION

Pharmacotherapy is the main form of treatment of RA; therefore, drug resistance induced by activated lymphocytes is a potentially serious challenge in the clinical management of RA. P-gp overexpression on activated pathogenic lymphocytes leads to the development of P-gp-mediated multidrug resistance. Therefore, treatments that target P-gp and preferentially reduce P-gp-mediated drug-resistance, can overcome drug-resistance. Taken together, we propose that the level of P-gp expressed on peripheral lymphocytes measured in RA patients is a useful marker for P-gp-mediated drug resistance and might help in the selection of more effective treatment, including treatment that reduce P-gp expression (such as biological agents) and inhibit P-gp function (such as P-gp competitors).

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ORIGINAL ARTICLE

Retrospective Study

Association of insulin resistance with serum ferritin and aminotransferases-iron hypothesis

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Abstract

AIM: To investigate the relationship of iron indices with diabetes mellitus (DM) in those without hemochromatosis.

METHODS: This cross-sectional study examined data collected during the Third National Health and Nutrition Examination Survey (NHANES Ⅲ). Only those who fasted properly and were not anemic with transferrin saturation < 45% were included (n = 6849). Insulin sensitivity and beta cell function were calculated from fasting glucose and insulin concentrations. Indices of iron metabolism were examined in the presence or absence of DM. We examined the relationship of insulin sensitivity and beta cell function with serum ferritin concentration. The influence of C-reactive protein and liver enzymes was also investigated.

RESULTS: Serum ferritin concentration was significantly higher in diabetic subjects (P = 0.0001 to < 0.000001). The difference remained significant after adjustment for age, body mass index, alcohol consumption, and mineral/iron supplement (P = 0.03 to < 0.000001). In those who did not take insulin, serum ferritin concentration was negatively associated with insulin sensitivity (P = 0.05 to 0.00001), but not with beta cell function. The alanine aminotransferase was correlated with serum ferritin concentration (P = 0.02 to < 0.000001) but not with insulin sensitivity, suggesting the role of the liver in iron-associated insulin resistance.

CONCLUSION: As most of diabetes is type 2 diabetes and insulin resistance is a cardinal feature of type 2 diabetes, disordered iron metabolism could play a role in the pathogenesis of insulin resistance and type 2 diabetes through its effect on liver function.

Key words: Diabetes mellitus; Insulin sensitivity; Beta cell function; Ferritin; Liver

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Core tip: Hemochromatosis and excess iron load has been implicated to play a role in the pathogenesis of diabetes mellitus. Serum ferritin concentration was significantly higher in diabetic subjects. Serum ferritin concentration was negatively associated with insulin sensitivity, but not with beta cell function. The association of alanine aminotransferase correlated with serum ferritin concentration, but not insulin sensitivity, suggesting the role of the liver in iron-associated insulin resistance. Disordered iron metabolism could play a role in the pathogenesis of insulin resistance and type 2 diabetes mellitus through its effect on liver function.

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INTRODUCTION

Diabetes mellitus (DM) is a common manifestation (53%-80%) of hereditary hemochromatosis[1], which is an autosomal recessive disorder caused by mutations in a gene designated HFE (OMIM: 235200). A mutation, C282Y, was detected in 83% of the patients, while it was only found in 3.2% of control chromosomes[2]. However, the allelic frequencies of C282Y mutation were similar between diabetic and control groups (6.3% *vs* 5.5%) in a large population from the United Kingdom[3]. Meta-analysis of published studies showed no evidence for over-representation of this allele in patients with type 2 diabetes[3]. Therefore, the C282Y mutation does not play a role in the pathogenesis of type 2 diabetes. Nevertheless, the role of iron metabolism in the pathogenesis of diabetes in the general population has been suggested in many cross-sectional studies[4-7]. Furthermore, a nested case-control study suggested a potential interaction between the HFE genotypes and heme iron in relation to the risk of type 2 diabetes[8].

In hereditary hemochromatosis, both insulin resistance and impaired insulin secretion have been suggested to play a role in its pathogenesis[9]. The role of insulin resistance in patients with secondary hemochromatosis from thalassemia major has been reported, while an additional defect in beta cell secretion cannot be excluded[10]. The association of serum ferritin concentration and insulin resistance has been reported in various liver diseases[11,12]. Furthermore, the underlying mechanism of iron-associated abnormal glucose homeostasis in the general population is not well understood.

To examine the role of iron in the pathogenesis of diabetes, we investigated the iron indices and the relative influence of an inflammatory marker and liver enzymes on glucose homeostasis in a nationally representative survey, third National Health and Nutrition Examination Survey (NHANES Ⅲ).

MATERIALS AND METHODS

Ethics statement

The National Center for Health Statistics of the Centers for Disease Control and Prevention conducted the NHANES Ⅲ in the United States from 1988 through 1994. This survey was designed to assess the health and nutrition status of a large representative sample in the United States. The survey and data collection was approved by the NHANES Institutional Review Board and documented consent was obtained from participants. Analysis of de-identified data from the survey is exempt from the federal regulations for the protection of human research participants. Only de-identified data from the survey was used in this study.

Study design and study sample

Detailed descriptions of the survey and the analytical methods of various assays have been published[13] and are also available at its website (http://www.cdc.gov/nchs/about/major/nhanes/datalink.htm#NHANESIII).

Race and ethnicity were self-reported by the participants. NHANES III was designed to provide reliable information from three major racial/ethnic groups: Non-Hispanic whites (NHW), non-Hispanic blacks (NHB), and Hispanics. The 4th group was excluded from this analysis for its small sample size and for encompassing a heterogeneous racial/ethnic group. There were 15021 subjects who had serum ferritin, fasting glucose and insulin concentration measured. Proper fasting is required to define diabetes status and to calculate insulin sensitivity and beta cell function from the fasting samples[14,15]. Only those who fasted for ⩾ 8 h and ⩽ 16 h were included (n = 7701). We excluded 180 subjects with hemoglobin < 11 g/dL, which is frequently associated with iron deficiency and falsely low HbA_{1C}. Since hemochromatosis is an established cause of diabetes, those with transferrin saturation ⩾ 45 were also excluded[16] (n = 672), which identified 98% of iron-overloaded subjects[17].

Ascertainment of DM

Diabetes was defined as a fasting glucose concentration ⩾ 126 mg/dL (7.0 mmol/L) or a 2-h postchallenged glucose concentration ⩾ 200 mg/dL (11.1 mmol/L)[14]. Without the 2-h postchallenged glucose concentration, the diagnosis of diabetes is frequently missed in those with elevated 2-h postchallenged glucose concentrations and normal fasting glucose concentrations[18]. However, only 3010 subjects had 2-h postchallenged glucose concentration measured. Their HbA_{1C} was very well correlated with 2-h postchallenged glucose concentration (r = 0.7558, P < 0.000001) and 2-h postchallenged glucose concentration of 200 mg/dL (11.1 mmol/L) was equivalent to HbA_{1C} of 6.3%. Therefore, we also defined diabetes in those with HbA1C ⩾ 6.3%.

Calculation of beta cell function and insulin sensitivity

Beta cell function (%B) and insulin sensitivity (%S) were calculated based on the homeostasis model assessment (HOMA)[15,19].

%B=(20 × fasting insulin concentration in mU/L)/(fasting glucose concentration in mmol/L - 3.5).

%S = 22.5/(fasting insulin concentration in mU/L × fasting glucose concentration in mmol/L).

Those with fasting glucose concentration < 3.5 mmol/L were excluded from analysis, since they had negative %B (n = 9). %B and %S obtained from the HOMA had been shown to correlate very well with the measured beta cell function and insulin sensitivity from various methods[15,20-22]. A quantitative insulin sensitivity check index (QUICKI), which had been shown to correlate with the measured insulin sensitivity by hyperinsulinemic euglycemic clamp very well[23], was also used.

$QUICKI=1/[\log_{10}(\text{fasting insulin concentration in mU/L}) + \log_{10}(\text{fasting glucose concentration in mg/dL})]$.

All of these methods have been validated in both non-diabetic subjects and diabetic subjects who did not take insulin[21-23]. Those who took insulin were excluded from these analyses (n = 51).

Statistical analysis

General descriptive variables were expressed as means ± SD. Since gender and ethnicity could potentially affect both iron metabolism and glucose homeostasis, the data were analyzed separately by gender and ethnic groups. Continuous variables were compared using two-tail Student t test between two groups or Analysis of Variance for more than two groups. Continuous data were expressed as means with 95%CI. Analysis of variance was used to examine the influence of covariates [age and body mass index (BMI)] on continuous variables between two groups. Least square regression analysis was used to investigate the relationship between two continuous variables. The influence of covariates (age, BMI, alcohol consumption, and mineral/iron intake) was also accounted for least square regression analysis. To further assess the association of serum ferritin concentration with estimated beta function and insulin sensitivity indices as well as the association of liver aminotransferases and C-reactive protein (CRP) with serum ferritin concentration and estimated insulin sensitivity indices, we also examine the trend across the quintile of serum ferritin concentration, liver aminotransferases and CRP. The comparisons were also adjusted for age, BMI, alcohol consumption, and mineral/iron intake. All the analyses were conducted in SYSTAT 11, Systat Software, Inc., Point Richmond, California, United States. A P value less than 0.05 was considered significant.

RESULTS

Study populations

The clinical features of the studied subjects were shown by gender and ethnic groups in Table 1. Based on previously published upper reference ranges[24], in male participants, 8.2% had elevated aspartate aminotransferase (AST > 37 U/L) and 9.6% had elevated alanine aminotransferase (ALT > 40 U/L) and in female participants, 7.7% had elevated AST (> 37 U/L) and 7.1% had elevated ALT (> 37 U/L).

Comparison of indices of iron metabolism in the presence or absence of diabetes

Iron, total iron binding capacity (TIBC), transferrin saturation, and serum ferritin concentration were

Table 1 Clinical features of studied subjects

	Non-Hispanic whites		Non-Hispanic blacks		Hispanics	
	Male	Female	Male	Female	Male	Female
n	1373	1602	896	1057	957	964
Age (yr)	55 ± 19	54 ± 20	43 ± 17	42 ± 16	42 ± 17	41 ± 17
Systolic blood pressure (mmHg)	130 ± 18	125 ± 21	128 ± 18	122 ± 20	125 ± 17	120 ± 20
Diastolic blood pressure (mmHg)	76 ± 10	72 ± 9	79 ± 11	74 ± 11	76 ± 10	71 ± 10
Body mass index (kg/m^2)	26.75 ± 4.63	26.39 ± 5.80	26.74 ± 5.34	29.07 ± 7.22	27.10 ± 4.64	28.26 ± 5.97
Transferrin saturation (%)	27.93 ± 8.39	24.58 ± 8.78	26.22 ± 8.11	21.75 ± 8.51	28.23 ± 8.59	22.95 ± 8.98
Ferritin (mcg/L)	176 ± 142	95 ± 104	215 ± 170	93 ± 111	169 ± 142	67 ± 95
Aspartate aminotransferase (U/L)	22 ± 8	19 ± 8	25 ± 17	22 ± 8	27 ± 16	23 ± 19
Alanine aminotransferase (U/L)	18 ± 11	14 ± 10	19 ± 13	18 ± 11	28 ± 24	21 ± 23
Gamma glutamyl transferase (U/L)	35 ± 55	21 ± 20	46 ± 59	35 ± 55	46 ± 53	30 ± 31
C-reactive protein (mg/dL)	0.43 ± 0.76	0.45 ± 0.62	0.48 ± 0.79	0.53 ± 0.65	0.44 ± 0.84	0.53 ± 0.80

Data presented mean ± SD.

Table 2 Comparison of serum indices of iron by the presence or absence of diabetes mellitus

	Non-Hispanic whites				Non-Hispanic blacks				Hispanics			
	Male		Female		Male		Female		Male		Female	
	DM	Non-DM	DM	Non-DM	DM	Non-DM	DM	Non-DM	DM	NOn-DM	DM	Non-DM
n	166	1207	137	1465	98	798	106	951	115	842	132	832
Iron (mcg/dL)												
Mean	93	95	83	87	82	87	70	75	90	98	82	84
(95%CI)	(89, 97)	(94, 97)	(78, 88)	(86, 89)	(77, 87)	(85, 89)	(64, 75)	(74, 77)	(84, 97)	(96, 100)	(76, 87)	(82, 86)
P^1	NS		NS		NS		NS		0.01		NS	
P^2	NS		NS		NS		NS		NS		NS	
P^3	NS		NS		NS		NS		NS		NS	
Total iron binding capacity (mcg/dL)												
Mean	351	344	359	360	333	334	342	353	353	354	362	377
(95%CI)	(343, 359)	(341, 346)	(350, 368)	(357, 363)	(322, 344)	(330, 337)	(331, 352)	(349, 356)	(343, 363)	(351, 358)	(351, 373)	(372, 381)
P^1	NS		NS		NS		NS		NS		0.01	
P^2	0.002		NS		NS		NS		NS		NS	
P^3	NS		NS		NS		NS		NS		NS	
Transferrin saturation (%)												
Mean	27	28	23	25	25	26	21	22	26	28	23	23
(95%CI)	(26, 28)	(28, 29)	(22, 25)	(24, 25)	(23, 26)	(26, 27)	(19, 22)	(21, 22)	(24, 28)	(27, 28)	(22, 25)	(22, 23)
P^1	NS		NS		NS		NS		0.03		NS	
P^2	NS		NS		NS		NS		NS		NS	
P^3	NS		NS		NS		NS		NS		NS	
Ferritin (mcg/L)												
Mean	228	169	152	84	282	206	167	86	230	150	138	56
(95%CI)	(200, 256)	(161, 177)	(129, 175)	(79, 89)	(237, 327)	(195, 218)	(139, 195)	(79, 92)	(190, 270)	(142, 159)	(104, 171)	(52, 60)
P^1	< 0.000001		< 0.000001		0.00003		< 0.000001		< 0.000001		< 0.000001	
P^2	0.0001		0.000003		0.02		0.0001		0.00001		< 0.000001	
P^3	0.0002		0.000003		0.03		0.0001		0.000004		< 0.000001	

Data presented mean with 95%CI. To convert values for iron and total iron binding capacity to mol/L, multiply by 0.1791. [1]*P* values for unadjusted comparison; [2]*P* values for comparison after adjustment for age and body mass index; [3]*P* values for comparison after adjustment for age, body mass index, alcohol consumption, and mineral/iron supplement. NS: Not significant ($P > 0.05$); DM: Diabetes mellitus; NDM: Non-diabetic mellitus.

compared between those with or without diabetes (Table 2). No consistent results were observed for iron, TIBC, and transferrin saturation, while serum ferritin concentration was markedly higher in diabetic than in non-diabetic subjects in all six groups. Diabetic subjects were older than non-diabetic subjects by 14-16 years ($P < 0.000001$) and were also more obese than non-diabetic subjects per BMI by 2.40-4.81 kg/m^2 ($P < 0.000001$). The difference in ferritin concentration between diabetic and non-diabetic subjects remained significant after adjustment of age, BMI, alcohol intake, and mineral/iron supplement. Thus, diabetes was associated with elevated serum ferritin concentration.

Association of serum ferritin concentration with beta cell function and insulin sensitivity

Diabetes results from an imbalance between beta cell function and insulin sensitivity. Thus, serum ferritin concentration could potentially be associated with either beta cell function, insulin sensitivity, or both. No association between %B and serum ferritin concentration was found after adjustment for both age and BMI (Table 3). In contrast, serum ferritin concentration was negatively associated with %S and

Table 3 Estimated beta cell function and insulin sensitivity indices by quintile of serum ferritin concentrations

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P^1	P^2	P^3
Beta cell function by the homeostasis model assessment (%B)[4]								
Non-Hispanic white males	103 (96, 110)	98 (92, 105)	97 (90, 104)	106 (94, 118)	113 (102, 124)	NS	NS	NS
Non-Hispanic white females	117 (110, 124)	115 (108, 122)	109 (103, 114)	111 (104, 119)	110 (102, 118)	NS	NS	NS
Non-Hispanic black males	113 (104, 122)	118 (107, 130)	112 (103, 120)	144 (107, 181)	117 (106, 129)	NS	NS	NS
Non-Hispanic black females	177 (156, 197)	178 (161, 196)	151 (137, 164)	135 (121, 150)	135 (123, 147)	0.000001	NS	NS
Hispanic males	104 (91, 118)	112 (101, 124)	106 (97, 114)	123 (106, 141)	128 (116, 140)	0.004	NS	NS
Hispanic females	152 (140, 165)	150 (135, 164)	140 (129, 150)	136 (124, 147)	130 (119, 141)	0.003	NS	NS
Insulin sensitivity by the homeostasis model assessment (%S)[5]								
Non-Hispanic white males	0.503 (0.472, 0.535)	0.549 (0.513, 0.585)	0.518 (0.484, 0.553)	0.494 (0.463, 0.525)	0.421 (0.388, 0.455)	0.00005	NS	0.05
Non-Hispanic white females	0.643 (0.610, 0.677)	0.603 (0.570, 0.637)	0.574 (0.539, 0.609)	0.555 (0.519, 0.591)	0.455 (0.420, 0.491)	< 0.000001	0.04	0.02
Non-Hispanic black males	0.562 (0.516, 0.608)	0.553 (0.504, 0.601)	0.559 (0.512, 0.605)	0.555 (0.498, 0.611)	0.48 (0.426, 0.535)	0.04	NS	0.05
Non-Hispanic black females	0.510 (0.476, 0.545)	0.479 (0.437, 0.522)	0.453 (0.421, 0.485)	0.458 (0.422, 0.495)	0.379 (0.343, 0.414)	0.000001	0.02	0.007
Hispanic males	0.538 (0.495, 0.580)	0.533 (0.489, 0.578)	0.527 (0.472, 0.583)	0.524 (0.460, 0.588)	0.338 (0.307, 0.370)	< 0.000001	0.01	0.007
Hispanic females	0.523 (0.482, 0.563)	0.535 (0.484, 0.587)	0.464 (0.426, 0.502)	0.444 (0.403, 0.485)	0.321 (0.289, 0.352)	< 0.000001	0.005	0.003
Insulin sensitivity by the simple QUICKI[6]								
Non-Hispanic white males	0.339 (0.336, 0.342)	0.343 (0.340, 0.346)	0.340 (0.336, 0.343)	0.337 (0.334, 0.341)	0.327 (0.323, 0.331)	< 0.000001	0.008	0.002
Non-Hispanic white females	0.352 (0.349, 0.355)	0.348 (0.345, 0.351)	0.345 (0.342, 0.348)	0.343 (0.340, 0.346)	0.33 (0.327, 0.334)	< 0.000001	0.00005	0.00001
Non-Hispanic black males	0.344 (0.339, 0.348)	0.342 (0.338, 0.347)	0.343 (0.339, 0.348)	0.341 (0.335, 0.346)	0.333 (0.328, 0.338)	0.003	0.03	0.01
Non-Hispanic black females	0.340 (0.336, 0.343)	0.335 (0.331, 0.339)	0.333 (0.329, 0.337)	0.333 (0.329, 0.337)	0.322 (0.317, 0.326)	< 0.000001	0.0006	0.0001
Hispanic males	0.341 (0.336, 0.345)	0.34 (0.336, 0.345)	0.339 (0.335, 0.344)	0.337 (0.332, 0.342)	0.318 (0.314, 0.322)	< 0.000001	0.0001	0.00001
Hispanic females	0.340 (0.336, 0.344)	0.340 (0.336, 0.345)	0.334 (0.330, 0.338)	0.330 (0.326, 0.335)	0.315 (0.310, 0.319)	< 0.000001	0.00003	0.00002

Data presented mean with 95%CI. [1]*P* values for trend, unadjusted; [2]*P* values for trend, after adjustment for age and body mass index; [3]*P* values for trend, after adjustment for age, body mass index, alcohol consumption, and mineral/iron supplement; [4]%B = (20 × fasting insulin concentration in mU/L) / (fasting glucose concentration in mmol/L - 3.5). Those with negative %B were excluded from analysis; [5]%S = 22.5/(fasting insulin concentration in mU/L × fasting glucose concentration in mmol/L); [6]Quicki = 1/[log(fasting glucose concentration in mg/dL) + log(fasting insulin concentration in mU/L)]. NS: Not significant; QUICKI: Quantitative insulin sensitivity check index.

QUICKI in all six groups. This relationship persisted after adjustment for age, BMI, alcohol consumption, and mineral/iron supplement. Therefore, we concluded that ferritin concentration was negatively associated with insulin sensitivity.

Role of inflammation in association between serum ferritin concentration and insulin sensitivity

In addition to reflection of the body iron store, serum ferritin is also an acute reactant. To explore the role of inflammation on the observed correlation, we examined the relationship of these indices with a marker of inflammation, CRP. No consistent association of CRP with either %S or QUICKI was observed (Table 4). A positive association between CRP and serum ferritin concentration was observed in NHW (both males and females), non-Hispanic black females, and Hispanic females, but not in non-Hispanic black. The associations remained unchanged after adjustment for age, BMI, alcohol intake, and mineral/iron supplement. Therefore, inflammation could not provide a uniform explanation for the underlying mechanism of the observed correlation between serum ferritin concentration and insulin sensitivity.

Role of the liver in association between serum ferritin concentration and insulin sensitivity

Elevated liver enzymes could result from iron deposition in the liver. To examine the role of the liver in the association of serum ferritin concentration with insulin sensitivity, we investigated the association of liver enzymes with serum ferritin concentration and insulin sensitivity. We focused on AST or SGOT, gamma glutamyl transpeptidase (GGT), and ALT or SGPT.

Table 4 Serum ferritin concentration and estimated insulin sensitivity indices by quintile of inflammatory marker - C-reactive protein

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P^1	P^2	P^3
Serum ferritin concentration (mcg/L)								
Non-Hispanic white males	142 (130, 155)	157 (144, 170)	179 (164, 195)	201 (180, 222)	201 (181, 221)	< 0.000001	0.000002	0.000005
Non-Hispanic white females	19 (18, 20)	49 (48, 50)	120 (116, 125)	152 (134, 171)	108 (95, 120)	0.000003	0.00003	0.00003
Non-Hispanic black males	186 (163, 209)	187 (167, 207)	267 (236, 298)	227 (202, 253)	254 (281, 228)	NS	NS	NS
Non-Hispanic black females	90 (74, 106)	79 (66, 91)	77 (65, 89)	102 (87, 118)	115 (98, 132)	0.002	0.0009	0.001
Hispanic males	150 (134, 166)	157 (135, 179)	161 (137, 185)	157 (136, 178)	172 (154, 191)	NS	NS	NS
Hispanic females	52 (44, 60)	56 (48, 64)	63 (53, 72)	72 (60, 84)	92 (68, 115)	0.00001	0.007	0.006
Insulin sensitivity by the homeostasis model assessment (%S)[4]								
Non-Hispanic white males	0.541 (0.505, 0.578)	0.531 (0.500, 0.562)	0.546 (0.509, 0.582)	0.459 (0.425, 0.494)	0.412 (0.383, 0.440)	< 0.000001	0.05	0.03
Non-Hispanic white females	0.684 (0.651, 0.718)	0.627 (0.592, 0.661)	0.596 (0.558, 0.634)	0.494 (0.461, 0.526)	0.422 (0.392, 0.452)	< 0.000001	0.004	0.004
Non-Hispanic black males	0.562 (0.517, 0.607)	0.572 (0.526, 0.618)	0.561 (0.499, 0.623)	0.549 (0.488, 0.611)	0.478 (0.426, 0.530)	0.05	NS	NS
Non-Hispanic black females	0.524 (0.489, 0.558)	0.524 (0.487, 0.560)	0.497 (0.464, 0.530)	0.404 (0.369, 0.438)	0.327 (0.288, 0.365)	< 0.000001	0.004	0.002
Hispanic males	0.558 (0.507, 0.608)	0.484 (0.438, 0.530)	0.525 (0.472, 0.578)	0.513 (0.460, 0.566)	0.372 (0.334, 0.411)	0.00001	NS	NS
Hispanic females	0.538 (0.495, 0.582)	0.53 (0.481, 0.579)	0.471 (0.430, 0.513)	0.392 (0.355, 0.429)	0.353 (0.319, 0.387)	< 0.000001	NS	NS
Insulin sensitivity by the simple QUICKI[5]								
Non-Hispanic white males	0.342 (0.339, 0.346)	0.342 (0.339, 0.345)	0.342 (0.339, 0.346)	0.332 (0.328, 0.336)	0.328 (0.325, 0.331)	< 0.000001	0.01	0.008
Non-Hispanic white females	0.356 (0.354, 0.359)	0.351 (0.348, 0.354)	0.347 (0.343, 0.350)	0.336 (0.333, 0.340)	0.328 (0.324, 0.331)	< 0.000001	0.004	0.005
Non-Hispanic black males	0.345 (0.341, 0.349)	0.345 (0.340, 0.349)	0.341 (0.336, 0.346)	0.34 (0.334, 0.345)	0.332 (0.327, 0.338)	0.01	NS	NS
Non-Hispanic black females	0.341 (0.337, 0.345)	0.341 (0.337, 0.345)	0.339 (0.335, 0.342)	0.327 (0.323, 0.331)	0.314 (0.310, 0.319)	< 0.000001	0.000002	0.000001
Hispanic males	0.342 (0.338, 0.347)	0.335 (0.331, 0.340)	0.338 (0.333, 0.343)	0.338 (0.333, 0.342)	0.321 (0.317, 0.326)	< 0.000001	NS	NS
Hispanic females	0.342 (0.338, 0.346)	0.34 (0.336, 0.344)	0.334 (0.329, 0.338)	0.325 (0.321, 0.329)	0.319 (0.314, 0.323)	< 0.000001	0.02	0.03

Data presented mean with 95%CI. [1]*P* values for trend, unadjusted; [2]*P* values for trend, after adjustment for age and body mass index; [3]*P* values for trend, after adjustment for age, body mass index, alcohol consumption, and mineral/iron supplement; [4]%S = 22.5/(fasting insulin concentration in mU/L × fasting glucose concentration in mmol/L); [5]Quicki = 1/[log(fasting glucose concentration in mg/dL) + log(fasting insulin concentration in mU/L)]. NS: Not significant; QUICKI: Quantitative insulin sensitivity check index.

The association of AST with %S and QUICKI was only noted in Hispanic males, but not in other 5 groups (Table 5). Adjustment for age, BMI, alcohol consumption, and mineral/iron supplement had no impact on the results. In contrast, a very close association was noted between AST and ferritin concentration in all 6 groups. Since AST is present in many tissues, including heart, skeletal muscle, kidney, and brain, we could not exclude the role of the liver based on no association between AST and insulin sensitivity in some groups.

GGT is a very sensitive indicator of hepatobiliary diseases and is found predominately throughout the hepatobiliary system, but also in other tissues. It was negatively associated with %S and QUICKI in all 6 groups (Table 6). The association remained after adjustment for age, BMI, alcohol consumption, and mineral/iron supplement. It was positively associated with serum ferritin concentration after adjustment for age and BMI. Therefore, the relationship of GGT with insulin sensitivity and serum ferritin concentration could provide a mechanistic insight of the liver in the association between insulin sensitivity and serum ferritin concentration.

The primary source of ALT is the liver. It was negatively associated with both %S and QUICKI in all six groups, and remained highly significant regardless of the adjustment for age, BMI, alcohol consumption, and mineral/iron supplement (Table 7). This relationship indicated an association of insulin resistance and liver diseases. Serum ferritin concentration was positively associated with ALT and also this association remained significant regardless of adjustment for age, BMI, alcohol consumption, and mineral/iron supplement. Since ALT is an indicator of liver diseases, a positive association between ALT and serum ferritin concentration suggests that increased iron deposition

Table 5 Serum ferritin concentration and estimated insulin sensitivity indices by quintile of aspartate aminotransferase

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P^1	P^2	P^3
Serum ferritin concentration(mcg/L)								
Non-Hispanic white males	148 (134, 162)	154 (140, 167)	180 (165, 196)	170 (155, 186)	228 (205, 251)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic white females	58 (51, 65)	61 (55, 68)	85 (76, 94)	102 (91, 112)	140 (122, 158)	0.0004	0.02	0.02
Non-Hispanic black males	175 (155, 196)	203 (178, 228)	197 (173, 222)	227 (203, 251)	267 (236, 298)	0.05	0.003	0.004
Non-Hispanic black females	84 (71, 96)	77 (66, 88)	80 (67, 92)	95 (81, 109)	127 (106, 149)	0.00001	0.004	0.005
Hispanic males	139 (124, 153)	134 (118, 151)	149 (134, 163)	155 (132, 177)	223 (194, 251)	< 0.000001	< 0.000001	< 0.000001
Hispanic females	47 (39, 56)	49 (42, 55)	59 (50, 68)	79 (57, 101)	101 (86, 115)	< 0.000001	0.00001	0.00002
Insulin sensitivity by the homeostasis model assessment (%S)[4]								
Non-Hispanic white males	0.491 (0.457, 0.526)	0.517 (0.484, 0.550)	0.497 (0.466, 0.528)	0.506 (0.471, 0.541)	0.478 (0.441, 0.514)	NS	NS	NS
Non-Hispanic white females	0.575 (0.544, 0.607)	0.597 (0.559, 0.630)	0.591 (0.554, 0.628)	0.555 (0.521, 0.588)	0.506 (0.470, 0.543)	0.02	NS	NS
Non-Hispanic black males	0.512 (0.465, 0.559)	0.531 (0.479, 0.584)	0.528 (0.486, 0.569)	0.557 (0.508, 0.605)	0.561 (0.499, 0.623)	NS	NS	NS
Non-Hispanic black females	0.447 (0.410, 0.484)	0.452 (0.418, 0.487)	0.472 (0.437, 0.506)	0.461 (0.424, 0.499)	0.443 (0.401, 0.485)	NS	NS	NS
Hispanic males	0.501 (0.460, 0.542)	0.55 (0.495, 0.604)	0.524 (0.473, 0.574)	0.477 (0.424, 0.530)	0.404 (0.359, 0.449)	< 0.000001	< 0.000001	< 0.000001
Hispanic females	0.472 (0.430, 0.514)	0.48 (0.443, 0.516)	0.468 (0.430, 0.507)	0.49 (0.442, 0.539)	0.374 (0.330, 0.418)	0.006	NS	NS
Insulin sensitivity by the simple QUICKI[5]								
Non-Hispanic white males	0.337 (0.333, 0.340)	0.34 (0.337, 0.343)	0.338 (0.335, 0.341)	0.338 (0.334, 0.342)	0.334 (0.330, 0.338)	NS	NS	NS
Non-Hispanic white females	0.346 (0.343, 0.349)	0.347 (0.344, 0.350)	0.347 (0.343, 0.350)	0.343 (0.340, 0.346)	0.336 (0.332, 0.340)	0.009	NS	NS
Non-Hispanic black males	0.337 (0.332, 0.342)	0.339 (0.334, 0.344)	0.341 (0.337, 0.345)	0.343 (0.338, 0.348)	0.3417 (0.336, 0.346)	NS	NS	NS
Non-Hispanic black females	0.331 (0.327, 0.335)	0.332 (0.328, 0.336)	0.335 (0.332, 0.339)	0.333 (0.329, 0.337)	0.33 (0.326, 0.334)	NS	NS	NS
Hispanic males	0.337 (0.333, 0.342)	0.342 (0.337, 0.346)	0.338 (0.333, 0.343)	0.333 (0.328, 0.338)	0.325 (0.320, 0.329)	< 0.000001	< 0.000001	< 0.000001
Hispanic females	0.334 (0.329, 0.338)	0.336 (0.332, 0.340)	0.334 (0.330, 0.338)	0.335 (0.331, 0.340)	0.32 (0.315, 0.325)	0.0001	NS	NS

Data presented mean with 95%CI. [1]*P* values for trend, unadjusted; [2]*P* values for trend, after adjustment for age and body mass index; [3]*P* values for trend, after adjustment for age, body mass index, alcohol consumption, and mineral/iron supplement; [4]%S = 22.5/(fasting insulin concentration in mU/L × fasting glucose concentration in mmol/L); [5]Quicki = 1/[log(fasting glucose concentration in mg/dL) + log(fasting insulin concentration in mU/L). NS: Not significant; QUICKI: Quantitative insulin sensitivity check index.

in the liver is associated with liver dysfunction. Furthermore, a positive association between ALT and serum ferritin concentration and a negative association between ALT and insulin sensitivity suggests a negative association of serum ferritin concentration with insulin sensitivity as we had observed.

DISCUSSION

To examine the role of iron metabolism in diabetes, the indices of iron metabolism were compared in patients with and without diabetes. We found that subjects with diabetes had a higher serum ferritin concentration than those without diabetes. To explore the underlying pathophysiology, we observed that serum ferritin concentration was negatively associated with insulin sensitivity (%S and QUICKI), but not with beta cell function. Therefore, a high serum ferritin concentration is associated with insulin resistance and is a risk factor for DM.

Since ferritin contains the second largest pool of iron in the body next to hemoglobin[16], serum ferritin concentration closely correlates with total body iron stores, mainly in the liver[26]. In this study, serum ferritin concentration is negatively associated with insulin sensitivity, suggesting an association of insulin resistance with total body iron stores. However, it is well known that ferritin is also an acute phase reactant[26]. To further explore this issue, we examined the correlation of CRP, an inflammatory marker[27], with insulin sensitivity and serum ferritin concentration. Without a consistent result across 3 ethic/racial groups and both genders, we concluded that in this study, no consistent relationship of CRP with either insulin sensitivity or serum ferritin concentration was observed in all 6 groups. Furthermore, in this population only

Table 6 Serum ferritin concentration and estimated insulin sensitivity indices by quintile of gamma glutamyl transferase

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P^1	P^2	P^3
Serum ferritin concentration (mcg/L)								
Non-Hispanic white males	141 (125, 157)	146 (131,161)	174 (156, 191)	179 (162, 196)	227 (204, 250)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic white females	55 (48, 62)	70 (61, 78)	95 (81, 110)	92 (77, 107)	137 (121, 154)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic black males	165 (144, 187)	191 (163, 219)	221 (195, 247)	220 (194, 246)	298 (263, 333)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic black females	60 (45, 74)	79 (68, 91)	84 (67, 100)	97 (83, 112)	141 (119, 163)	< 0.000001	< 0.000001	< 0.000001
Hispanic males	105 (93, 117)	137 (121, 153)	152 (132, 172)	173 (144, 202)	240 (208, 272)	< 0.000001	< 0.000001	< 0.000001
Hispanic females	38 (30, 47)	38 (32, 44)	59 (47, 70)	82 (70, 93)	109 (90, 129)	< 0.000001	< 0.000001	< 0.000001
Insulin sensitivity by the homeostasis model assessment (%S)[4]								
Non-Hispanic white males	0.622 (0.584, 0.659)	0.521 (0.486, 0.556)	0.5061 (0.465, 0.546)	0.422 (0.387, 0.458)	0.402 (0.367, 0.436)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic white females	0.732 (0.695, 0.769)	0.648 (0.609, 0.686)	0.587 (0.547, 0.626)	0.48 (0.444, 0.516)	0.397 (0.361, 0.433)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic black males	0.607 (0.560, 0.654)	0.588 (0.522, 0.653)	0.537 (0.491, 0.583)	0.489 (0.429, 0.548)	0.462 (0.404, 0.520)	0.0001	NS	0.05
Non-Hispanic black females	0.554 (0.515, 0.594)	0.454 (0.419, 0.489)	0.45 (0.409, 0.491)	0.407 (0.372, 0.442)	0.383 (0.332, 0.434)	< 0.000001	0.0002	0.00002
Hispanic males	0.67 (0.625, 0.715)	0.562 (0.486, 0.638)	0.423 (0.383, 0.463)	0.439 (0.376, 0.501)	0.359 (0.299, 0.419)	< 0.000001	0.00005	0.000005
Hispanic females	0.623 (0.569, 0.677)	0.494 (0.448, 0.539)	0.424 (0.381, 0.466)	0.338 (0.301, 0.375)	0.343 (0.298, 0.389)	< 0.000001	0.000002	0.000003
Insulin sensitivity by the simple QUICKI[5]								
Non-Hispanic white males	0.351 (0.348, 0.354)	0.341 (0.338, 0.344)	0.339 (0.335, 0.342)	0.328 (0.324, 0.332)	0.325 (0.321, 0.330)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic white females	0.361 (0.358, 0.364)	0.353 (0.350, 0356)	0.347 (0.344, 0.350)	0.335 (0.332, 0.339)	0.323 (0.319, 0.327)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic black males	0.349 (0.345, 0.353)	0.344 (0.338, 0.350)	0.342 (0.337, 0.346)	0.334 (0.328, 0.339)	0.331 (0.325, 0.337)	< 0.000001	0.01	0.004
Non-Hispanic black females	0.345 (0.341, 0.348)	0.334 (0.330, 0.338)	0.332 (0.328, 0.337)	0.327 (0.323, 0.332)	0.321 (0.315, 0.326)	< 0.000001	< 0.000001	< 0.000001
Hispanic males	0.356 (0.352, 0.360)	0.341 (0.335, 0.347)	0.330 (0.325, 0.334)	0.329 (0.323, 0.334)	0.318 (0.312, 0.324)	< 0.000001	< 0.000001	< 0.000001
Hispanic females	0.351 (0.346, 0.355)	0.338 (0.334, 0.342)	0.329 (0.325, 0.334)	0.318 (0.313, 0.323)	0.316 (0.311, 0.322)	< 0.000001	< 0.000001	< 0.000001

Data presented mean with 95%CI. [1]*P* values for trend, unadjusted; [2]*P* values for trend, after adjustment for age and body mass index; [3]*P* values for trend, after adjustment for age, body mass index, alcohol consumption, and mineral/iron supplement; [4]%S = 22.5/(fasting insulin concentration in mU/L × fasting glucose concentration in mmol/L); [5]Quicki = 1/[log(fasting glucose concentration in mg/dL) + log(fasting insulin concentration in mU/L)]. NS: Not significant; QUICKI: Quantitative insulin sensitivity check index.

1.28% (range: 0.64% in Hispanic males to 1.95% in non-Hispanic black males) of the participants had an elevated CRP ⩾ 3 mg/L, which is the 90th percentile for healthy young adults[28]. After exclusion of those with a CRP ⩾ 3 mg/L, association of CRP with insulin sensitivity was only observed in NHW females (P = 0.006 for %S and P = 0.00002 for QUICKI) and NHB females (P = 0.03 for %S and P = 0.0001 for QUICKI) and correlation of CRP with ferritin concentration was only observed in NHW females (P = 0.005), NHB females (P = 0.001), and MA females (P = 0.01), after adjustment for age and BMI. Therefore, it is very unlikely that inflammation is the underlying mechanism for the observed negative association between serum ferritin concentration and insulin sensitivity.

Next we examined the role of the liver on the observed association between insulin sensitivity and serum ferritin concentration. Among these three liver enzymes, AST is the least specific liver maker and ALT is the most liver specific marker. All three liver enzymes were correlated with serum ferritin concentration very well, suggesting a closed association between elevated serum ferritin and liver dysfunction. However, we observed the different strengths of the correlation of insulin sensitivity across three liver enzymes. Among them, AST is the least specific for the liver diseases, the association between AST and insulin sensitivity only observed in Hispanic male group. In contrast, a negative association of ALT and GGT with insulin sensitivity was observed in all 6 groups. The negative correlation of liver enzymes with insulin sensitivity indicates the role of hepatic dysfunction in insulin resistance. Therefore, these observations imply the role of iron-associated elevated ALT and GGT in the pathogenesis of insulin resistance. The role of the liver in the pathogenesis of DM is well-established[29,30]. Furthermore, the relation-

Table 7 Serum ferritin concentration and estimated insulin sensitivity indices by quintile of alanine aminotransferase

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P^1	P^2	P^3
Serum ferritin concentration (mcg/L)								
Non-Hispanic white males	147 (132, 162)	169 (153, 185)	168 (152, 183)	177 (159, 194)	220 (200, 241)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic white females	71 (63, 79)	71 (61, 82)	86 (77, 95)	95 (84, 107)	123 (107, 139)	0.05	0.02	0.02
Non-Hispanic black males	168 (147, 189)	187 (165, 210)	201 (177, 225)	247 (219, 275)	267 (238, 296)	0.01	0.00005	0.00005
Non-Hispanic black females	80 (67, 92)	79 (68, 89)	87 (73, 100)	98 (81, 114)	120 (100, 140)	0.00003	0.000004	0.000005
Hispanic males	128 (113, 143)	128 (116, 140)	142 (126, 157)	172 (144, 200)	229 (206, 253)	< 0.000001	< 0.000001	< 0.000001
Hispanic females	42 (35, 49)	70 (48, 93)	57 (49, 65)	64 (55, 73)	101 (87, 116)	< 0.000001	0.000009	0.000009
Insulin sensitivity by the homeostasis model assessment (%S)[4]								
Non-Hispanic white males	0.551 (0.517, 0.584)	0.544 (0.511, 0.578)	0.512 (0.476, 0.549)	0.483 (0.448, 0.518)	0.399 (0.370, 0.429)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic white females	0.634 (0.602, 0.666)	0.628 (0.591, 0.664)	0.583 (0.546, 0.622)	0.55 (0.519, 0.582)	0.429 (0.395, 0.463)	0.00002	0.004	0.002
Non-Hispanic black males	0.63 (0.576, 0.684)	0.576 (0.522, 0.630)	0.564 (0.514, 0.615)	0.478 (0.423, 0.524)	0.44 (0.397, 0.482)	0.00002	0.003	0.003
Non-Hispanic black females	0.527 (0.490, 0.563)	0.498 (0.462, 0.534)	0.462 (0.424, 0.500)	0.427 (0.389, 0.465)	0.363 (0.330, 0.396)	< 0.000001	< 0.000001	< 0.000001
Hispanic males	0.635 (0.569, 0.700)	0.57 (0.525, 0.615)	0.48 (0.430, 0.530)	0.448 (0.410, 0.486)	0.324 (0.295, 0.352)	< 0.000001	< 0.000001	< 0.000001
Hispanic females	0.55 (0.509, 0.591)	0.528 (0.484, 0.571)	0.468 (0.427, 0.509)	0.409 (0.365, 0.452)	0.331 (0.295, 0.368)	< 0.000001	0.000003	0.000002
Insulin sensitivity by the simple QUICKI[5]								
Non-Hispanic white males	0.344 (0.341, 0.347)	0.343 (0.339, 0.346)	0.339 (0.335, 0.342)	0.335 (0.332, 0.339)	0.326 (0.322, 0.329)	< 0.000001	< 0.000001	< 0.000001
Non-Hispanic white females	0.352 (0.349, 0.355)	0.35 (0.347, 0.345)	0.346 (0.342, 0.349)	0.343 (0.340, 0.346)	0.327 (0.324, 0.331)	0.000001	0.0005	0.0002
Non-Hispanic black males	0.349 (0.344, 0.354)	0.344 (0.339, 0.349)	0.343 (0.338, 0.348)	0.335 (0.330, 0.339)	0.33 (0.326, 0.335)	0.00005	0.01	0.009
Non-Hispanic black females	0.341 (0.337, 0.345)	0.338 (0.334, 0.342)	0.333 (0.329, 0.337)	0.33 (0.326, 0.334)	0.321 (0.316, 0.325)	< 0.000001	< 0.000001	< 0.000001
Hispanic males	0.348 (0.343, 0.353)	0.345 (0.341, 0.349)	0.333 (0.328, 0.338)	0.332 (0.328, 0.336)	0.317 (0.313, 0.321)	< 0.000001	< 0.000001	< 0.000001
Hispanic females	0.343 (0.339, 0.347)	0.34 (0.336, 0.344)	0.334 (0.330, 0.338)	0.327 (0.323, 0.331)	0.315 (0.311, 0.320)	< 0.000001	< 0.000001	< 0.000001

Data presented mean with 95%CI. [1]*P* values for trend, unadjusted; [2]*P* values for trend, after adjustment for age and body mass index; [3]*P* values for trend, after adjustment for age, body mass index, alcohol consumption, and mineral/iron supplement; [4]%S = 22.5/(fasting insulin concentration in mU/L × fasting glucose concentration in mmol/L); [5]Quicki = 1/[log(fasting glucose concentration in mg/dL) + log(fasting insulin concentration in mU/L)]. NS: Not significant; QUICKI: Quantitative insulin sensitivity check index.

ship of elevated ALT concentration with diabetes and insulin resistance has been reported[31]. In Pima Indians, elevated ALT was associated with hepatic insulin resistance but not with whole body insulin resistance or beta cell function[32]. Insulin sensitivity obtained from the HOMA as used in this study, has been demonstrated to be correlated with hepatic insulin sensitivity[33]. Thus from the observations in this study, iron could play a role in hepatic insulin resistance.

The role of serum ferritin concentration in diabetes[4] has been examined in this population. Elevated ferritin concentration has been reported to be associated with an increased risk of diabetes, but the role of inflammation could not be excluded[4]. Elevated serum ferritin concentration also has been reported to be associated with insulin resistance[34]. In the present study, we confirmed the association of serum ferritin concentration with DM and insulin sensitivity assessed by both %S and QUICKI. Although the association of inflammation and insulin resistance has been demonstrated in this population[35], we demonstrated that the inflammatory hypothesis is not likely the underlying mechanism of the reported associations in this study. In addition, from the observed associations of ALT and GGT with serum ferritin concentration and insulin sensitivity, we provided the evidence suggesting that the role of liver in the pathogenesis of iron-associated insulin resistance.

Iron-induced oxidant stress has proposed to play a key role in iron-mediated tissue damage[36-38]. Although the molecular events of iron-mediated tissue damage have not been fully elucidated, mitochondria are the targets of iron-mediated damage and iron may be preferentially toxic to cells with high mitochondrial activity[39], such as hepatocytes and pancreatic beta cells. Impaired mitochondrial activity has been observed in the insulin-resistant offspring of patients with type

2 diabetes[40] and mitochondrial defect can also lead to the metabolic syndrome[41]. Therefore, iron-induced oxidative stress with mitochondrial dysfunction could be one of the underlying mechanisms in these metabolic disorders.

Because of the cross-sectional nature of the study, a temporal relationship and the biological basis of the association between serum ferritin concentration and these metabolic disorders could not be established. However, our observations have some bearing on the plausible mechanisms. Furthermore, the caustic role of iron in these processes is suggested by interventional studies. In patients with clinical evidence of non-alcoholic fatty liver disease, quantitative phlebotomy induced iron depletion to a level of near-iron deficiency results in a 40%-55% improvement of both fasting and glucose-stimulated plasma insulin concentrations and near-normalization of ALT[42]. Quantitative phlebotomy also leads to improvement in insulin sensitivity in a group of subjects with high-ferritin type 2 diabetes[43]. Therefore, iron could be the culprit of these conditions.

The current sample set did provide enough information to distinguish type 1 and type 2 diabetes. However, as 95% of diabetes is type 2 diabetes and insulin resistance is a cardinal feature of type 2 diabetes, the current study is most applicable to type 2 diabetes. Our observations are consistent with the published results[44-46] with some new clinical implications. In this study, even without clinical evidence of iron overload, iron could be associated with liver damage and insulin resistance. A clinical trial of quantitative phlebotomy in the subjects with elevated ferritin concentration is warranted to test this hypothesis. Once it is demonstrated, quantitative phlebotomy could be recommended for those patients with DM or insulin resistance, who also have elevated serum ferritin concentration. Although the underlying molecular mechanism of the association remains to be elucidated, our observations imply that the liver could play a role in iron-associated insulin resistance.

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COMMENTS

Background

Liver has been noted to play a role in the pathogenesis of type 2 diabetes. Hemochromatosis and excess iron load has been implicated to play a role in the pathogenesis of type 2 diabetes. Iron metabolism could a role in the pathogenesis of type 2 diabetes.

Research frontiers

Diabetes mellitus is a common manifestation of hemochromatosis. Although the common mutation of *HFE* is not associated with type 2 diabetes, the iron metabolism could play a role in the pathogenesis of diabetes. In hemochromatosis, both insulin resistance and impaired insulin secretion have been suggested to play a role in its pathogenesis. However, the underlying mechanism of iron-associated abnormal glucose homeostasis in the general population is not well understood.

Innovations and breakthroughs

In this study, the authors found that subjects with diabetes had a higher serum ferritin concentration than those without diabetes. The authors also observed that serum ferritin concentration was negatively associated with insulin sensitivity (%S and QUICKI), but not with beta cell function. Therefore, a high serum ferritin concentration is associated with insulin resistance and is a risk factor for diabetes mellitus. The authors further demonstrated that the inflammatory hypothesis is not likely the underlying mechanism of the reported associations in this study. In addition, from the observed associations of alanine aminotransferase (ALT) and gamma glutamyl transpeptidase with serum ferritin concentration and insulin sensitivity, the authors provided the evidence suggesting that the role of liver in the pathogenesis of iron-associated insulin resistance. Iron-induced oxidant stress has proposed to play a key role in iron-mediated tissue damage and iron-induced oxidative stress with mitochondrial dysfunction could be one of the underlying mechanisms in these metabolic disorders.

Applications

As in patients with clinical evidence of non-alcoholic fatty liver disease, quantitative phlebotomy induced iron depletion to a level of near-iron deficiency results in a 40%-55% improvement of both fasting and glucose-stimulated plasma insulin concentrations and near-normalization of ALT and quantitative phlebotomy also leads to improvement in insulin sensitivity in a group of subjects with high-ferritin type 2 diabetes, a clinical trial of quantitative phlebotomy in the subjects with elevated ferritin concentration is warranted to test this hypothesis. Once it is demonstrated, quantitative phlebotomy could be recommended for those patients with diabetes mellitus or insulin resistance, who also have elevated serum ferritin concentration.

Terminology

Ferritin: Ferritin is a protein in the body that binds to iron; most of the iron stored in the body is bound to ferritin. In humans, it acts as a buffer against iron deficiency and iron overload. Serum ferritin concentration closely correlates with total body iron stores, mainly in the liver.

Peer-review

This is an interesting study. The authors have evaluated the relationship of iron with diabetes mellitus (DM) and concluded that disordered iron metabolism could play a role in the pathogenesis of insulin resistance and DM through its effect on liver function.

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How reliable is online diffusion of medical information targeting patients and families?

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Abstract

AIM: To determine whether online diffusion of the "Ten Warning Signs of Primary Immunodeficiency Diseases (PID)" adheres to accepted scientific standards.

METHODS: We analyzed how reproducible is online diffusion of a unique instrument, the "Ten Warning Signs of PID", created by the Jeffrey Modell Foundation (JMF), by Google-assisted searches among highly visited sites from professional, academic and scientific organizations; governmental agencies; and patient support/advocacy organizations. We examined the diffusion, consistency of use and adequate referencing of this instrument. Where applicable, variant versions of the instrument were examined for changes in factual content that would have practical impact on physicians or on patients and their families.

RESULTS: Among the first 100 sites identified by Google search, 85 faithfully reproduced the JMF model, and correctly referenced to its source. By contrast, the other 15 also referenced the JMF source but presented one or more changes in content relative to their purported model and therefore represent uncontrolled variants, of unknown origin. Discrepancies identified in the latter included changes in factual content of the original JMF list (C), as well as removal (R) and introduction (I) of novel signs (Table 2), all made without reference to any scientific publications that might account for the drastic changes in factual content. Factual changes include changes in

the number of infectious episodes considered necessary to raise suspicion of PID, as well as the inclusion of various medical conditions not mentioned in the original. Together, these changes will affect the way physicians use the instrument to consult or to inform patients, and the way patients and families think about the need for specialist consultation in view of a possible PID diagnosis.

CONCLUSION: The retrieved adaptations and variants, which significantly depart from the original instrument, raise concerns about standards for scientific information provided online to physicians, patients and families.

Key words: Information technology and human health; Expert consultation online; Online medical information; Warning signs; Infection; Diagnosis

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Core tip: We analyzed how reproducible is online diffusion of a unique instrument, the "Ten Warning Signs of Primary Immunodeficiency Diseases", to define whether information made available to physicians, families and support/advocacy groups through the internet adheres to accepted scientific standards. The results show that this instrument is diffused through many sites, but the actual scientific contents often depart substantially from their purported model. This raises concerns about the quality of scientific information provided online on medical matters, and on the need for corrective mechanisms in an age when the public is increasingly dependent on the internet as primary source of knowledge.

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INTRODUCTION

The personal computer and access to the Internet have affected medicine as rarely seen with previous technological advances, due to the large-scale inclusion of patients, families and support/advocacy groups as a highly motivated audience. Emerging online resources for communication, diffusion and education in the medical sciences profoundly changed the way physicians and patients view the possible diagnosis of uncommon, chronic disease, and the prospects for long and complex evaluation procedures. The Internet is now a major source of information on all aspects of medical science, which is consulted routinely by doctors worldwide. Many laymen also consult the Internet about rare, incapacitating diseases, and make decisions on the basis of factual information retrieved through Google and similar search tools. For families, this often means deciding for, or against, further search for specialty physicians and reference centers. Hence, online information must be consistent in itself, and based on best standards for scientific evidence, both in its initial presentation and in subsequent revised versions (whenever these are prompted by advances in medical knowledge). Deviance from this course will likely increase the risk of misinforming the public, entailing unpredictable risks for patients and their families.

The field of primary immunodeficiency diseases (PID) is a rapidly evolving area of medical science in which the advantages of diffusing medical information online are easy to grasp. PID[1] are a group of chronic, complex diseases[2] which poses a unique public health problem, because: (1) as a group, they are rare, but present worldwide; (2) they are biologically complex and very heterogeneous[3,4], but nevertheless share a set of major outcomes (increased susceptibility to repeated and/or opportunistic infections, autoimmunity and malignancy) which predispose to increased mortality and/or severe complications and sequelae[3]; (3) they arise in congenital defects of the immune system, which often lead to clinical deficiencies in infancy and childhood, and unless corrected through costly and technically complex procedures, may have a major impact on the growth and development, as well as socialization and schooling of the child[1-4]; and (4) they have a genetic basis that accounts for family clustering of cases, thereby making accurate diagnosis and increased awareness among health professionals indispensable for adequate family counseling[1-4].

In short, although PID patients must be managed by highly specialized professional teams, they mostly come from environments which lack specialists, and further lack the information about PID that would prompt families and primary care physicians to seek for specialists' counsel. Together, this creates the need for mechanisms to bring together the demographically rare, geographically dispersed and clinically heterogeneous patient population, on the one hand, and the institutionally rare, geographically concentrated and technologically demanding specialist population, on the other hand. This task can be made easier by letting the patients, their families and their primary care physicians know that they need the type of specialized care which is not available everywhere, but at specific tertiary institutions to which they can be referred. This challenge has been met by the Jeffrey Modell Foundation (JMF) through the elaboration of a list of Ten Warning Signs of Primary Immunodeficiency (hereafter referred to as "the JMF list"), to be diffused among patients and their families, primary care physicians and the general public, to raise awareness that certain clinical manifestations reflect an underlying PID that needs diagnostic workout. The creation of the JMF list has merit in itself, but its usefulness depends on whether it is scientifically accurate and correctly referenced, wherever it is found

online. Because this well-structured and authoritative text provides an ideal probe to examine whether medical information can be propagated online risk-free, we addressed here the online diffusion of the scientific information it embodies, as well as the emergence of variants that may appear in this process. The results show that the "Ten warning signs of primary immune deficiency" are quite suitable for an object-lesson on the fate of scientific information in the Cyberage.

MATERIALS AND METHODS

Study design

Initial evaluation of the frequency, extent and types of variation among online sites in the contents and referencing of the JMF list was carried out in the 100 most accessed web pages identified by Google with search term Ten warning signs of Primary Immune Deficiency (search date April 23, 2012). This was done to approximate as much as possible the most frequent situation, namely that in which someone tries, for the first time, to learn something about the Ten Warning Signs of PID online. To further assess the diffusion, accuracy of citation and consistency of use of the JMF list online, we examined in detail sites from: (1) professional, academic and scientific organizations, including, but not limited to, International Union of Immunological Societies, European Society of Immunodeficiencies, Sociedade Brasileira de Imunologia, Associação Brasileira de Alergia e Imunopatologia; (2) governmental agencies, including CDC and NIH; and (3) patient support organizations, including JMF and BRAGID (Grupo Brasileiro de Imunodeficiência).

Definition of terms

For the purposes of this study: (1) the concept is defined here as any list of Warning signs of PID, to be diffused among the nonspecialist public, regardless of whether it derives from the original JMF list; (2) the application of this Concept refers to the creation and use of any list of Warning signs of PID, which could be retrieved in our search; (3) referencing refers to the acknowledgement, by any given site applying the Concept, that it is aware of the JMF site (usually embodied in a link to the JMF site), or that JMF is considered a valid source of information on PID, regardless of whether the Concept as development in the site are actually related to the JMF list (*i.e.*, correct referencing) or not (incorrect referencing). Referencing, correct or incorrect, demonstrates that the site applying the Concept derives a measure of scientific credibility from acknowledging JMF as the referred source; and (4) variation refers to any departure in structure, language, or factual content (including quantitative information), from the original JMF list, which is accessible at the JMF site (www.jmfworld.com).

On the other hand, any variant list retrievable by the search tool could also originate in a completely independent source, unrelated to JMF, and in this case would represent an independent application of the Concept. If such a hypothetical alternative source were properly referenced, the application would not be included in our study as an undue variation of the JMF model. In our study, however (see results and discussion), both preserved and variant versions of the JMF list, some of the latter strikingly different from the JMF original, were all referenced to the JMF site.

Identification and classification of variants

Variation was considered significant if: (1) information contained in the original was removed (R), or changed (C) in factual/quantitative content so as to be of practical consequence; (2) information not contained in the original was introduced (I); and (3) information contained in the original was rearranged (displaced in its original context), or generalized (absorbed into a more comprehensive class not explicitly mentioned in the original) so as to change (C) the interpretation of the conveyed message. Deletion or addition of signs represents extreme examples of removal and introduction, respectively. A significant variant may differ from the standard in one or more signs, and therefore one significant change is sufficient to define a variant. On the other hand, preservation (P) of the original content is defined as complete identity of content between any given variant and the JMF list, and therefore precludes any significant change of content. Accordingly, less significant, or nonsignificant, variation, such as a change in language that did not affect the factual information, or a change in the order in which the warning signs were listed, was also found in our study, but was not further discussed below, to help us focus on discussing consequential issues.

The frequency of variation is defined as the ratio between the number of variant versions and the number of total versions sampled. The extent of variation (*i.e.*, the degree to which any given version differs from the purported original) found, as well as the types of variation detected (removal, introduction, change of content), can be found in the accompanying lists of sites which faithfully reproduce the JMF list (Table 1) and of variations found in those that do not (Table 2). For the extreme forms of introduction, which result in addition of novel signs, amounting to a drastic change in content, we further recorded the number of novel signs added to the original JMF material.

RESULTS

Preservation/change of the original message among highly accessed pages. Although numerous online information sources nominally refer to the JMF list, not every site consulted shows complete P of JMF original contents. The majority of pages faithfully reproduce the original information (Table 1), and are representative of adequate online sources of medical information (out of 48, 10 were from pharmaceutical companies; 21 from patient support groups focusing on severe and

Table 1 Identification of the sites with complete preservation of contents relative to the Jeffrey Modell Foundation list among the top 100 accessed by Google

www.jmfworld.com/
http://www.info4pi.org
http://www.pia.org.uk/publications/10_signs_of_pia
http://www.nichd.nih.gov/health/topics/primary_immunodeficiency.cfm
www.cisociety.com/files/what-is-PID.html
www.hizentra.com/docs/Hizentra_10_warning_signs_PIDD.pdf
http://www.justmommies.com/forums/f325-choosing-not-to-vaccinate/2004706-10-warning-signs-primary-immunodeficiency-psa-conjunction-cdc.html
http://pediatrics.about.com/od/primaryimmunodeficiency/Primary_Immunodeficiency.htm
https://www.mygardian.com/gardian/.../resources.html;.
http://www.allergyclinic.co.nz/guides/74.html
http://smr.newswire.ca/en/canadian-immunodeficiency-society/april-29-world-primary-immunodeficiency-day
http://www.gammagardliquid.com/professional-resources/order-tools-and-resources.html
http://www.treatingpi.com/primary-immunodeficiency-faq.aspx
http://www.uhhospitals.org/rainbowchildren/ourservices/allergyimmunology/tabid/3016/patientresources.aspx
http://smr.newswire.ca/en/canadian-immunodeficiency-society/april-29-world-primary-immunodeficiency-day
http://www.justmommies.com/forums/f325-choosing-not-to-vaccinate/2004706-10-warning-signs-primary-immunodeficiency-psa-conjunction-cdc.html
http://www.baxterbiotherapeutics.com/us/us_t_immunoglobulin.html
https://webforms.baxterbioscience.com/immune/order.jspa
http://allergistmommy.blogspot.com/2011/07/always-sick-could-it-be-immune.html
http://journals.lww.com/pidj/Citation/2011/10000/Clinical_Features_That_Identify_Children_With.6.aspx
https://www.wellpartner.com/SpecialtyRxFaqs.aspx
www.uhhospitals.org/.../immunodeficiency.pdf -
http://www.chw.org/display/PPF/DocID/36622/router.asp
http://www.biorx.net/brochures/10-Warning-Signs-of-PI.pdf
http://healthlibrary.stanford.edu/resources/bodysystems/immune_pid.html
http://www.pia.org.uk/gp_info.html
http://allergycases.org/2009/08/primary-immunodeficiency-disorders-pidd.html
http://www.savanna.k12.ok.us/Nurse/illustrated_poster_eng%20illustrated.pdf
http://www.isitpid.com/news_detail.cfm?e=7
http://ainotes.wikispaces.com/Immunodeficiency+Warning+Signs
http://nihrecord.od.nih.gov/newsletters/10_31_2000/story02.htm
http://www.allergyclinic.co.nz/whatsnew.html
http://www.babycenter.com/204_most-warning-signs-for-kids-8217-immune-disorders-are-off-ba_10349792.bc
http://www.gammaked.com/media/10_Warning_Signs_of_PI.pdf
http://www.disabled-world.com/disability/awareness/primary-immunodeficiency.php
https://plus.google.com/110859855629071891085/posts/5ijJ3nVYayp
http://www.ipopi.org/uploads/JMF%20Advocacy%20Toolkit_2011.pdf
http://books.google.com.br/books?id=H7GVhb27mo4C&pg=PA42&lpg=PA42&dq=10+Warning+Signs+of+Primary+Immunodeficiency&source=bl&ots=pz0rX9QK2E&sig=wKp2GGabmE0g7q_GRYB-vpwTt3g&hl=pt-BR&ei=iqOLTqCCBuXm0QGUrvX6BA&sa=X&oi=book_result&ct=result&resnum=5&ved=0CDgQ6AEwBDhQ#v=onepage&q=10%20Warning%20Signs%20of%20Primary%20Immunodeficiency&f=false
http://ezinearticles.com/comment.php?10-Warning-Signs-of-Primary-Immunodeficiency&id=1297957
http://dallasallergy.net/services/immunodeficiency
https://webforms.baxterbioscience.com/ggliquid/orderNew.jspa
http://www.doaj.org/doaj?func=abstract&id=467945
http://www.rare-diseases.eu/2010/IMG/Media/Czerniawska-PID%20Poster_Jeffrey%20Modell%20Foundation.pdf

chronic conditions; 4 mirrored the JMF site; 5 were from hospitals; 1 from an elementary school; 2 from NIH; 1 from an university; 1 from a scientific society; 3 reviewed a book on PID). Nevertheless, we found a sizeable minority (15 out of the 100 most highly accessed sites identified by Google) that displayed lists departing substantially from their purported model (Table 2). Discrepancies identified in the latter included changes in factual content of the original JMF list (C), as well as removal (R) and introduction (I) of novel signs (Table 2), all made without reference to any scientific publications that might account for the drastic changes in factual content.

Incorrect referencing in variant pages

Importantly, 14 of these 15 variant lists directly refer to the original JMF (direct links), or to sites with links to JMF (indirect links), as their source, and therefore represents undisputable examples of incorrect referencing, an unacceptable practice by current standards of scientific communication. Importantly, the variant versions include some sponsored by professional societies, governmental regulatory and research agencies, and respected biomedical research institutes, which thereby lend authority to an online resource that fails to meet standards of citation and referencing.

Major departures from the JMF list

Among the top 100 pages identified by Google, 3 formed a set with similar content, which departed strikingly from the JMF list. This included 1 page associated with the Mayo Clinic (http://www.mayoclinic.com/health/primary-immunodeficiency/DS01006), and 2 pages associated with www.livestrong.com (www.

Table 2 Variant versions of the Jeffrey Modell Foundation list in the first 100 pages retrieved by Google

	10 warning signs of primary immunodeficiency of the Jeffrey Modell Foundation										I		JMF	
Site consulted	1	#2	#3	#4	#5	#6	#7	#8	#9	#10	Y/N	*n*	Direct link[1]	Indirect link
a	C	P	P	P	P	P	C	P	C	P	N	0	N	N
b	C	P	P	R	P	P	C	P	C	P	N	0	Y	N
c	C	C	C	R	P	C	R	R	R	C	Y	4	N	Y
d	C	P	P	P	P	P	C	P	C	P	N	0	Y	N
e	C	P	P	P	P	P	C	P	C	P	N	0	Y	N
f	C	P	P	P	P	P	C	P	C	P	N	0	Y	N
g	C	C	P	P	P	P	C	R	C	P	Y	1	N	Y
h	C	P	R	P	P	P	C	P	C	P	Y	4	Y	N
i	C	P	P	P	P	P	C	P	C	P	N	0	N	Y
j	C	P	R	P	P	P	C	P	R	P	Y	4	Y	N
k	C	P	P	P	P	P	C	P	C	P	N	0	Y	N
l	C	P	P	P	P	P	C	P	C	P	N	0	N	Y
m	C	P	P	P	P	P	C	P	C	P	N	0	Y	N
n	C	P	P	P	P	P	C	P	C	P	N	0	N	Y
o	C	P	P	P	P	P	C	P	C	P	N	0	Y	N

[1]http://www.info4pi.org/aboutPI/index.cfm?section=aboutPI&content=warningsigns; [a]http://www.stopgettingsick.com/template.cfm-1685; [b]http://www.aafp.org/afp/2003/1115/p2011.html; [c]http://www.livestrong.com/article/71656-top-ten-warning-signs-primary/; [d]http://docphyl.com/index.php?x=pages/pimmunodeficiency; [e]http://ezinearticles.com/?10-Warning-Signs-of-Primary-Immunodeficiency&id=1297957; [f]http://www.momlogic.com/resources/primary_immunodeficiency_diseases.php; [g]http://www.irishhealth.com/article.html?id=3927; [h]http://www.aefat.es/docs/inmunodefprim_aafp.pdf; [i]www.bpl.co.uk/...primary-immunodeficiency/39; [j]http://www.aafp.org/afp/2003/1115/p2001.html; [k]http://www.insidetoronto.com/insidetoronto/article/57439; [l]http://www.allergyconsumerreview.com/sinus-infection.html; [m]http://www.aacijournal.com/content/7/S1/S11; [n]thegunnybag.files.wordpress.com/.../primary-im; [o]http://www.nichd.nih.gov/health/topics/primary_immunodeficiency.cfm. P: Preserved; R: Removed; I: Introduced; C: Changed content; *n*: Number of novel signs introduced. Search verified 04/23/2012.

livestrong.com/article/71656-top-ten-warning-signs-primary/; and www.livestrong.com/article/253355-10-signs-of-primary-immunodeficiency/), which presents itself as based on the Mayo Clinic. In this particular example, the key message was profoundly altered, but presented no support of scientific evidence to justify these changes. It also included a series of explanatory notes in language accessible to the nonmedical public, which represents a further departure from the language in which the JMF original is cast, of obvious consequence for the impact on families and patients, regardless of whether it is scientifically accurate. This specific example, which is of undeniable relevance, given the high number of accesses, as well as the high scientific profile of the sponsoring institution, and the wider audience presumably intended, illustrates the hazards of uncontrolled diffusion of medical information online.

Variation is not unconsequential

Scientific information was changed in some variant versions so as to have the potential for undesirable consequences in medical practice. A clear example follows: The "Four or more new ear infections within 1 year" of the JMF list were changed into "Eight or more new ear infections within 1 year" in several sites (The American Academy of Family Physicians; University of Wisconsin; the http://www.stopgettingsick.com/template.cfm-1685 patient support site; The Australasian Society of Clinical Immunology and Allergy/ASCIA). In the Mayo Clinic-related sites mentioned above, the same original content was generalized to "Frequent/recurrent infections". In the first case, a patient who would previously be considered worthy of evaluation for PID (according to the JMF list) must now suffer twice as many ear infections before qualifying as a candidate for PID evaluation. In the second case, the criteria became so broad as to lose their usefulness, for the definition would equally well apply to the numerous people living in conditions of poor hygiene and sanitation worldwide, who have no PID. The fate of the patient will therefore hinge on which version of the list has been consulted by the physician, an issue with broader implications for the rights of patients to appropriate medical attention.

A further example of generalization is provided by the Mayo Clinic, which introduced in their list of warning signs the following: inflammation and infection in internal organs, including the liver; autoimmune disorders, such as Lupus erythematosus, arthritis or type I diabetes; hematological disorders, such as low platelet counts and anemias; digestive problems, including cramps, loss of apetite, nausea and diarrhea; and delayed growth and development. Considering the high frequency with which any of the preceding occurs in the absence of PID, it is open to question whether such comprehensive enumeration of "warning signs for PID" will benefit patients and improve the physician's capabilities to deal with PID.

Local adaptations - is PID the same everywhere?

A different, but related, finding points to the adaptation of the original model to regional peculiarities. A very good example of adaptation is offered by BRAGID (Brazilian Group for Immune Deficiency), a patient-support organization. Although the BRAGID site (www.

imunopediatria.org.br) is not within the top 100 sites identified by Google, and therefore was not included in Table 2, it is here analyzed in detail because it plays a very important role in Brazil, where other sources of information on PID are scarce. The list accessed through BRAGID includes:"repeated intestinal infections, chronic diarrhea, severe asthma, collagen (autoimmune) diseases, adverse effects of BCG (tuberculosis) vaccination, mycobacterial infections, and a clinical phenotype suggestive of immune deficiency" (an odd item in a list directed to a public supposed to know little about immune deficiency and even less about the associated clinical phenotypes).

This adaptation likely reflects an effort to increase the usefulness of the concept to a developing country, where exposure to the mycobacteria that causes tuberculosis remains very important. The reference to adverse effects of BCG vaccination is equally appropriate to this context, since this is not a universally enforced practice, but is very relevant to Brazil as well as to PID patients[5-7]. However, the spectrum of clinical conditions enumerated includes repeated intestinal infections, chronic diarrhea, severe asthma and autoimmune diseases. This is less likely to help, as in a developing country such as Brazil infectious pathogens and environmental pollutants are major contributing factors for intestinal infection, chronic diarrhea, severe asthma, and even autoimmune manifestations in endemic diseases, which far outweigh their possible contribution to PID detection and management. As an effort of regional adaptation is to be commended, when justified by local factors, one should never omit the fact that this is an evolution from the original concept of signs of PID; by consequence, referencing to a well-established model without recording that it is a modification counters standard practice.

DISCUSSION

Anonymous expertise in an age of evidence-based medicine

It is surprising that factual content that informs medical decisions and medical education could undergo such uncontrolled variation online, especially in an age that stresses the importance of rigorous scientific documentation for sound medical practice. The fate of the JMF list online should raise concern in clinicians and scientists alike, because this unexplained variance in factual content counters basic rules in scientific communication, as significant changes in content were made without justification or references, and no responsibility was taken for them. Through incorrect referencing to the original from which they depart, variant lists are further misleading both physicians and patients as to the scientific basis of their content, and evading accountability for the possible untoward consequences of disseminating inaccurate information. Such attitude is common in many dimensions of the Cyberspace, since any private citizen with access to the Internet can upload content for all to view and download elsewhere, no matter how idiosyncratic or absurd this content may be. In domains other than medical science, the cover of anonimity also facilitates the dissemination of opinions that would probably be held back, if the author could easily be made accountable for them. Curiously, this sharply contrasts with the traditional environment in medical science, where authors typically identify themselves to the public, and highlight their professional qualifications and experience when delivering expert opinion, while referees tend to be covered by anonimity, although they act as guardians of scientific standards of originality, veracity and accountability. In an age of democratic online publishing, the provision of arbitrary, anonymous views as innocent "variations" of somebody else's better-known work does not further the cause of evidence-based medicine.

Academic freedom, scientific accountability and public trust

The JMF list of warning signs, like any scientific document, is perfectable and even open to challenge, a particularly timely issue, as illustrated by recent publications[8-10]. This work does not address the validity of the original list, but to show that it did not undergo online diffusion without a significant amount of distortion, adaptation or modification. Our intent is to highlight the potential presented by the Internet for distortion or corruption of factual information when nobody is paying attention to what is being diffused, and to who takes responsibility for the modifications.

While we concede that online medical information should be subject to no censorship, it is not sound science to advance a dissenting opinion which has neither an identifiable author nor a peer-reviewed reference. Academic freedom allows anyone to create his/her own list of warning signs of PID, or to modify an existing one if it is understood to be outdated. In the field of immunological diseases, which includes PID, a time-honored classification by Gell and Coombs has recently been revised in-depth, on the basis of extensive scientific evidence[11]. The latter revision was certainly not an anonymous document, nor lacked references to back it. By the same token, those willing to do so for lists of PID signs should take responsibility for the changes made, in addition to acknowledging their indebtedness to the original they improved upon, rather than attributing their own views to the JMF through incorrect referencing. The confidence of the public in medical information diffused online could only increase if unjustified changes to this information were reported to the original source, thereby promoting sound practices and scientific accountability.

We hope this examination of a strictly defined issue (variations of content in a single piece of medical information), which is highly appropriate as an object for study, but relevant to a relatively small number of people worldwide, will prompt others, better qualified

than ourselves, to extend our initial probing to broader areas, where issues may be manifold and potentially relevant to public health in a larger scale. Among the most interesting and timely subjects for such a broadened examination, we would suggest online resources focusing on other diseases such as AIDS and tuberculosis, which, like PID, involve issues of increased susceptibilityto infection, but have far greater social impact, due to the number of affected subjects worldwide.

COMMENTS

Background

Information technology and the Internet have profoundly changed the way physicians, patients, families and support/advocacy groups access and handle information on diseases, diagnoses, and novel/experimental treatments, but it remains unclear whether this revolutionary change adheres to accepted scientific standards of reliability, accuracy and referencing. The authors analyzed how reproducible is online diffusion of a unique instrument, the "Ten Warning Signs of primary immunodeficiency diseases (PID)". This was done because: (1) PID are a large and heterogeneous group of diseases, with related but distinct mechanisms, variable clinical presentations and complex therapeutic choices, requiring specialized diagnostic tools and expert counseling, not necessarily available in general hospitals and clinics; (2) the rarity of most varieties of PID, and the geographical concentration of the diagnostic and therapeutic resources, highlight the practical value of bringing together PID specialists and prospective patients through information diffused online; and (3) this instrument is clearly and concisely written, providing accurate, well-organized and useful information, therefore unreliable diffusion of its contents would have, in practice, a negative effect on the quality of information provided online.

Research frontiers

Although most people wouldn't think of the personal computer and the internet as advanced medical technology, they are fully integrated into medical practice in most countries, and the public, including doctors, trusts them to make important decisions. Nevertheless, there is no systematic follow-up of the contents of medical information pieces propagated in the internet. This is difficult to do in most cases, because large pieces of information on the same subjects would have to be compared, and there are many pieces to compare. In the authors' case, this examination is made easy, because the authors followed a very small piece of factual information up, which is well-structured and divided in numbered statements, so that comparison between the original and any variants is facilitated. This approach allowed the authors to examine, for the first time, the issue of uncontrolled fates for medical information online, and to evaluate the extent of the resulting distortions.

Innovations and breakthroughs

What is novel in the study is that, by examining the diffusion, consistency of use and adequate referencing of this instrument, among highly visited sites from professional, academic and scientific organizations, governmental agencies and patient support/advocacy organizations, the authors could show that the actual contents displayed often depart substantially from their purported model, in form and factual content, including unjustified eliminations and additions. In short, in a sizable proportion of cases, information is deleted, distorted or corrupted, but these changes go unnoticed or unreported.

Applications

The study shows that, by the appropriate choice of a piece of information to be monitored, it is possible to determine how well it adheres to accepted standards of scientific communication, which is central to its credibility and practical usefulness. This strategy should be applicable to other areas of Medicine affecting large numbers of people, as for instance those concerned by tuberculosis, AIDS or the benefits and risks of vaccination.

Terminology

PID are a large number of different diseases of variable clinical presentation and severity, and demanding specialized diagnosis and management which is not available everywhere. Due to their similarities in mechanisms and manifestations, PID as a group is associated with a constellation of clinical signs and symptoms, mostly due to a higher frequency of infection. The "Ten Warning Signs of PID" is short list of such signs and symptoms, intended to help families to decide whether or not a given patient should be evaluated for PID.

Peer-review

The authors have picked a usual yet interesting and important question regarding the diffusion of medical information in the cyberage.

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