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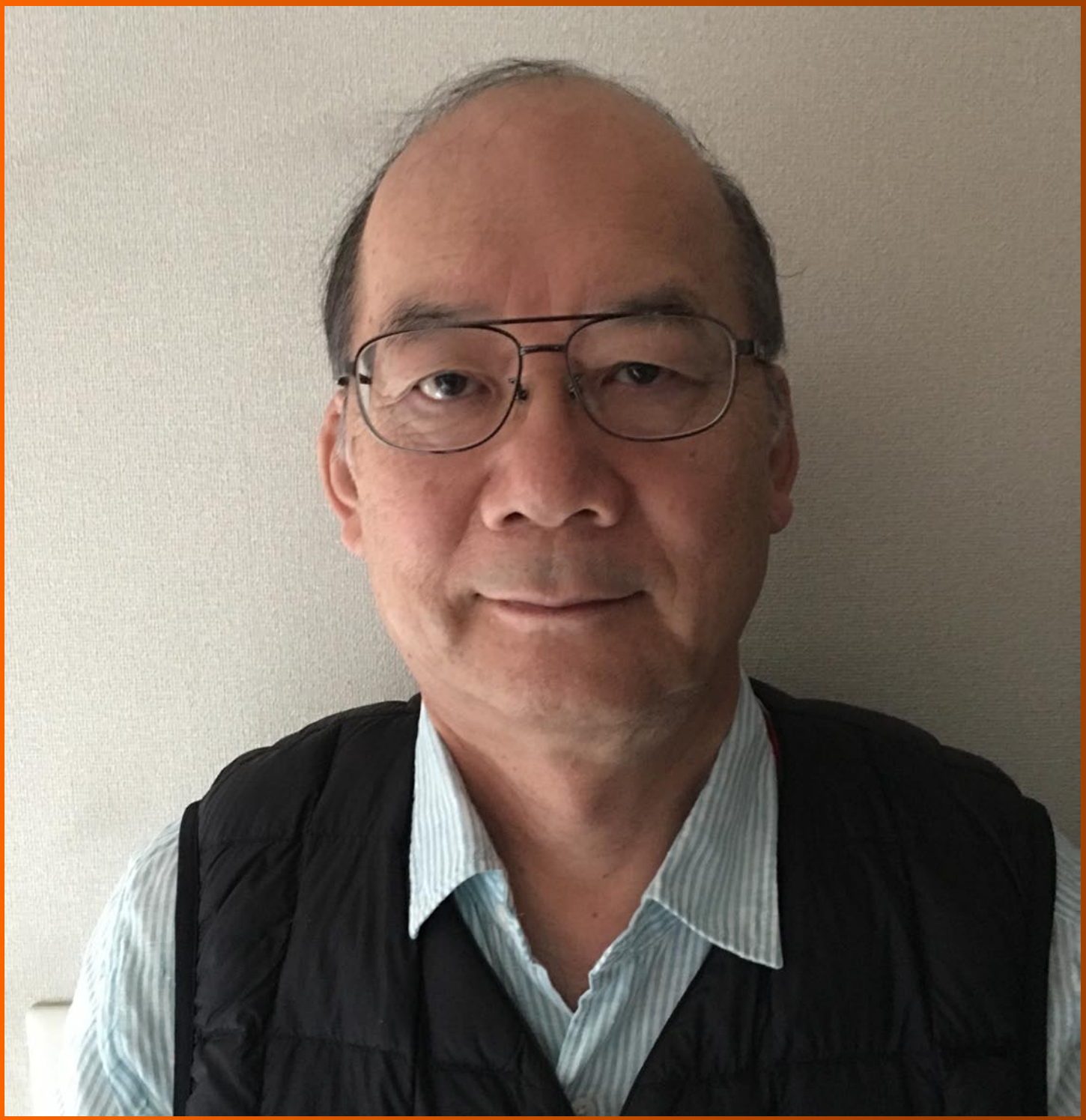

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EDITORIAL

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

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

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.

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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-

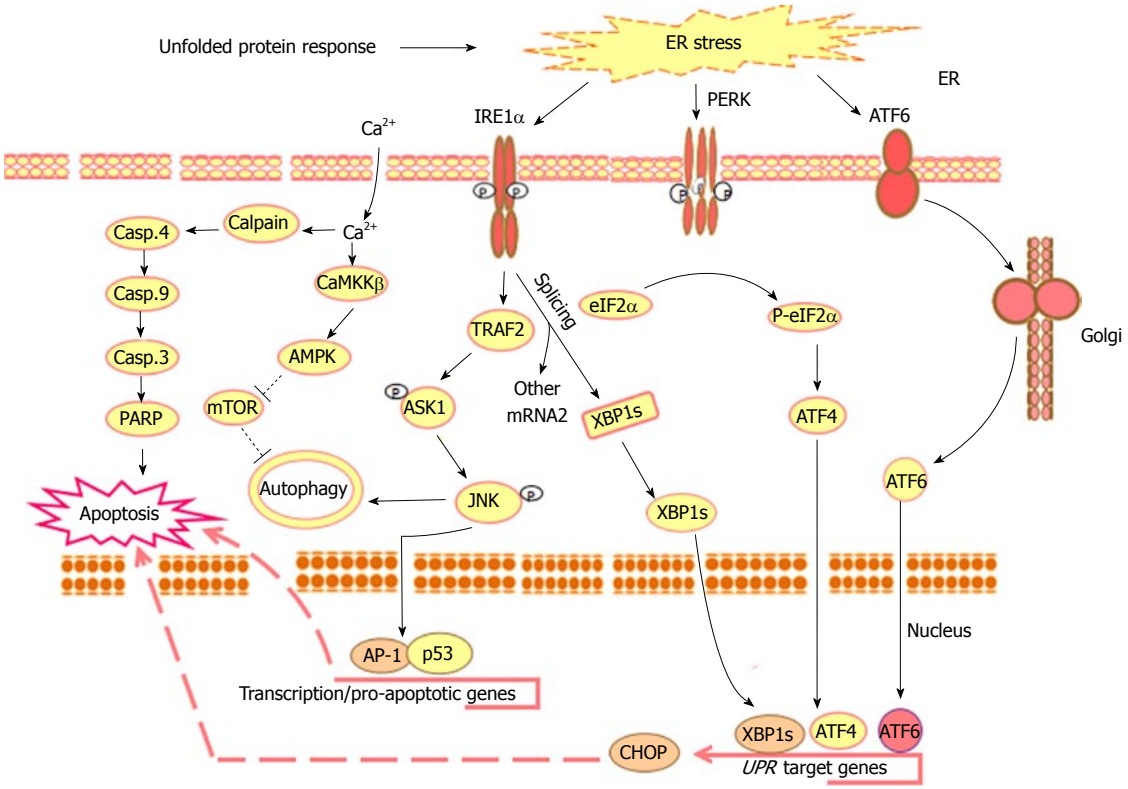

Figure 1 Outline of the main unfolded protein response-mediated mechanisms in response to the molecular action of anti-cancer agents. Upon the accumulation of misfolded proteins in the ER lumen, chaperones, the ER stress sensors PERK, IRE1α and ATF6 become active. The phosphorylation of PERK allows it 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

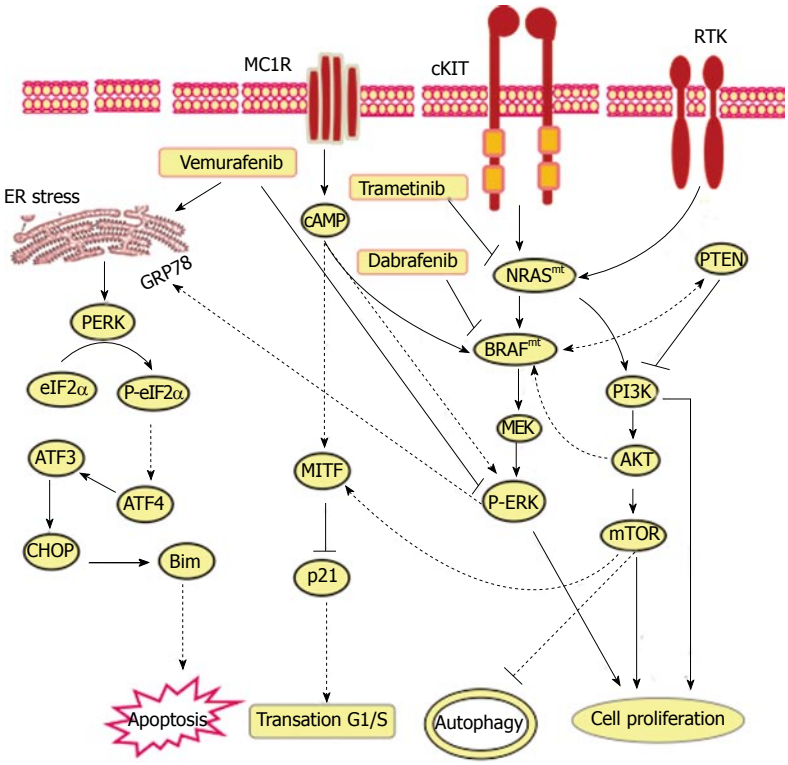

Figure 2 Proposed models for the mechanistic role of endoplasmic reticulum stress in the modulation of the anti-tumor efficiency of vemurafenib, dabrafenib and trametenib in melanoma patients harboring activating neuroblastoma RAS viral (v-ras) oncogene homolog or rapidly accelerated fibrosarcoma murine 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

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.

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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

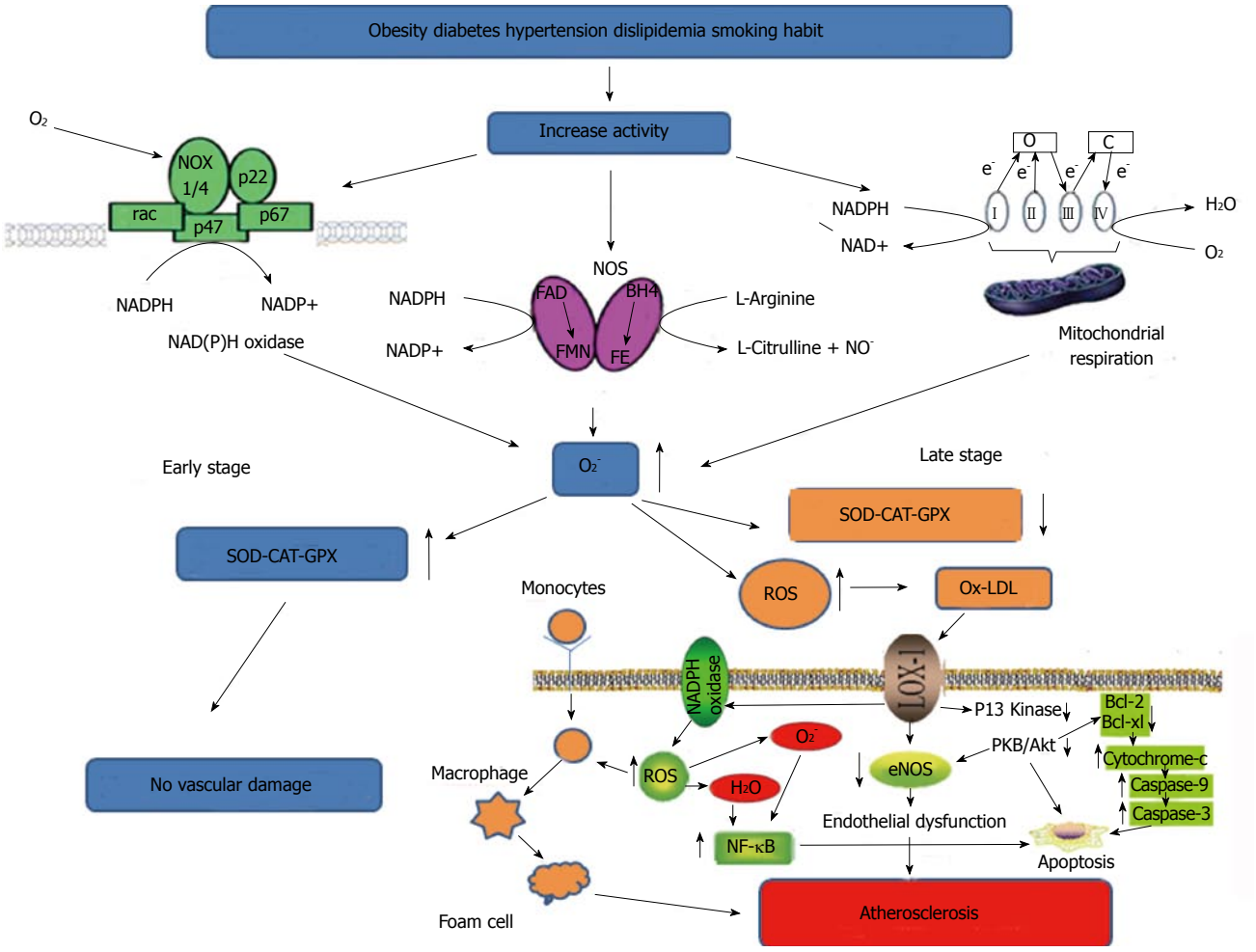

Figure 1 Biochemical events that favor the increase of reactive oxygen species. In the early stages of CAD, ROS do not cause damage due to the presence of an enzymatic compensatory mechanism. In late stage this mechanism is saturated and no longer allows an efficient defense, so that other biochemical 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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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

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

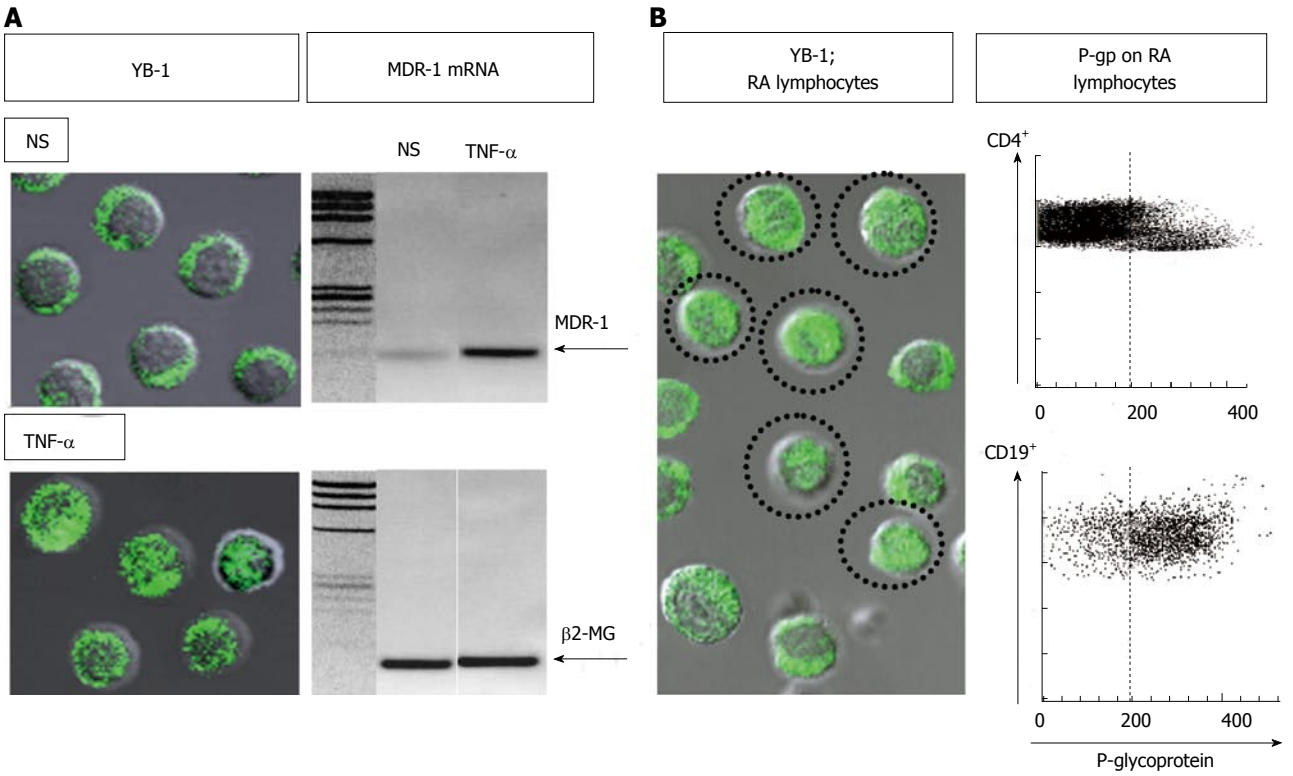

Figure 1 Up-regulation of nuclear translocation of Y-box-binding protein-1, transcription of multidrug resistance 1 in lymphocytes, and P-glycoprotein expression on lymphocytes. A: Left: Immunostaining and confocal microscopy analysis of Y-box-binding protein-1 (YB-1) in 1 × 10^5 of peripheral blood 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

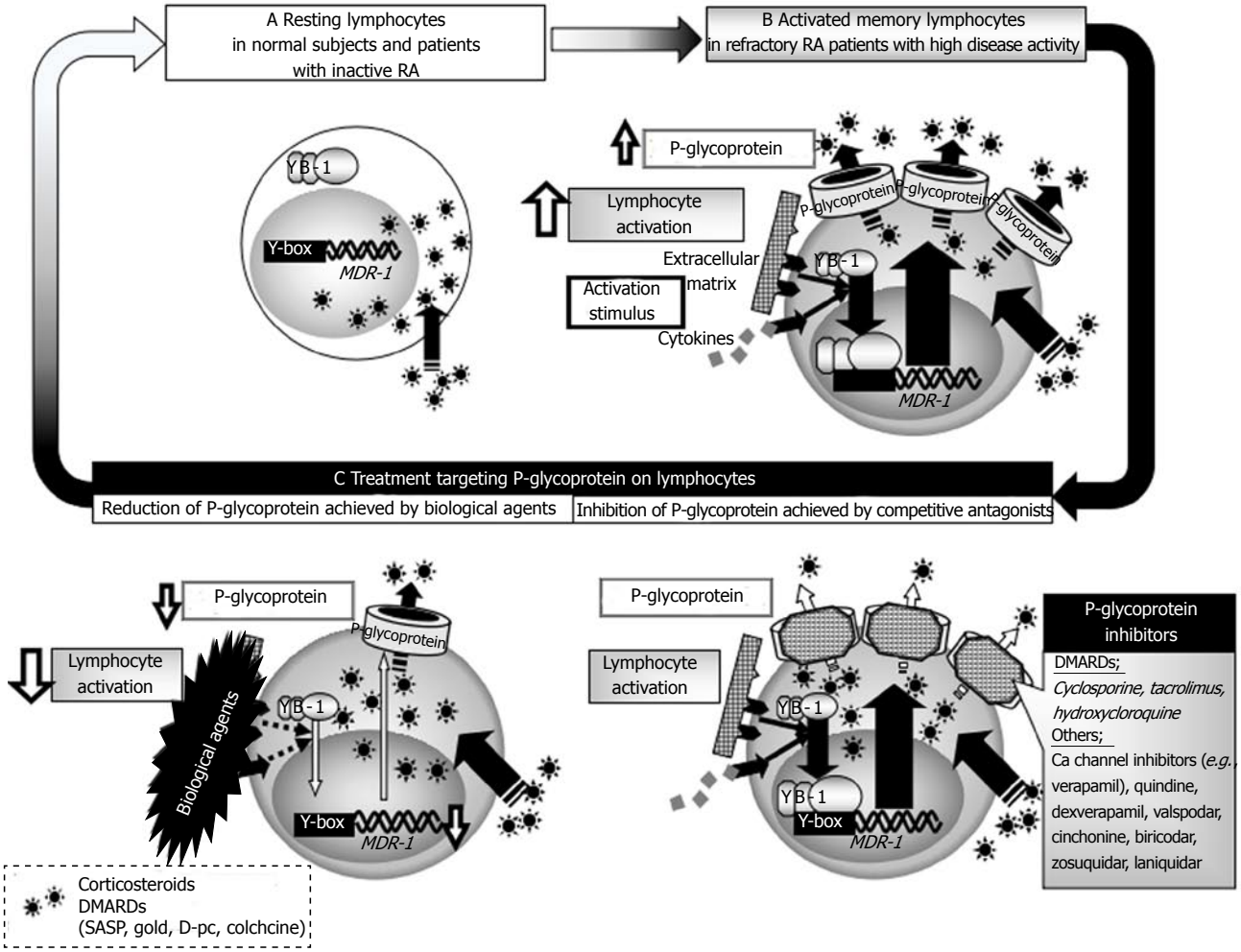

Figure 2 Schematic diagram of the relevance of P-glycoprotein to drug resistance in rheumatoid arthritis. A: Y-box-binding protein-1 is located in the cytoplasm of lymphocytes and P-glycoprotein (P-gp) is only marginally expressed on normal lymphocytes of normal subjects and patients with inactive RA; 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

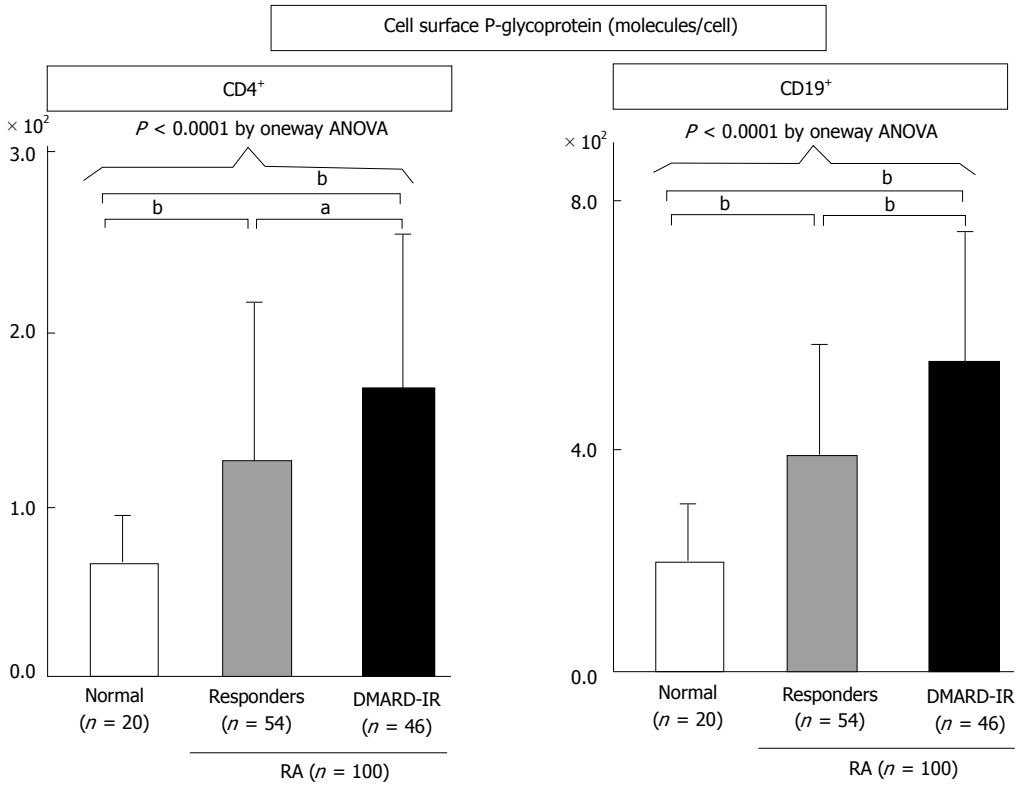

Figure 3 Expression of P-glycoprotein on lymphocytes from patients with refractory rheumatoid arthritis, as determined by flow cytometry. P-glycoprotein (P-gp) expression on $CD4^+$ or $CD19^+$ peripheral blood lymphocytes from 20 normal volunteers (open bar) and 100 RA patients [responders, hatched 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-

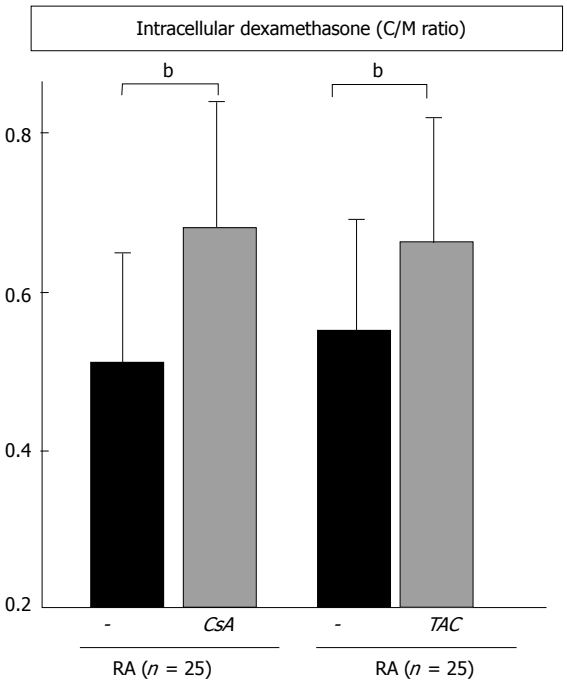

Figure 4 Inhibition of P-glycoprotein-related dexamethasone efflux by cyclosporine and tacrolimus. The C/M ratio in peripheral blood mononuclear cells of 25 rheumatoid arthritis (RA) patients was measured in the absence or presence of 100 ng/mL of cyclosporine (CsA), or 10 ng/mL of tacrolimus (TAC). 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 hydroxychloroquine (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

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

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.

Xavier-Elsas P, Bastos SE, Gaspar-Elsas MIC. How reliable is online diffusion of medical information targeting patients and families? *World J Exp Med* 2015; 5(4): 244-250 Available from: URL: http://www.wjgnet.com/2220-315X/full/v5/i4/244.htm DOI: http://dx.doi.org/10.5493/wjem.v5.i4.244

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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