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REVIEW

Endothelium-derived essential signals involved in pancreas organogenesis

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Abstract

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

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

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

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

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INTRODUCTION

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

ENDOTHELIUM AS AN ENDOCRINE ORGAN

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

Table 1 EC-derived factors related to organogenesis

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

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

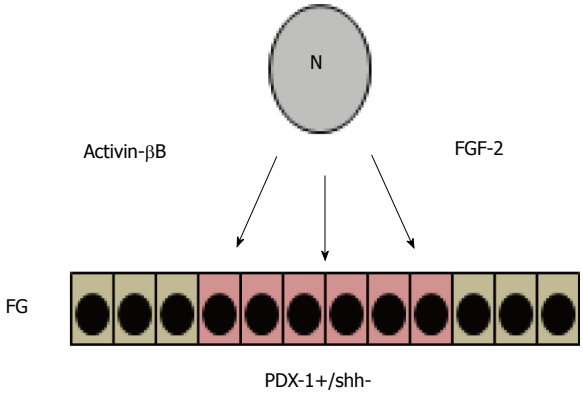

Figure 1 Early cell-cell interactions that give rise to pancreatic cells derived from definitive endoderm of the foregut (FG). Factors released by notochord (N) such as activin-βB and FGF-2 permit expression of PDX-1 and suppress expression of sonic hedgehog. FGF: Fibroblast growth factor; PDX: Pancreatic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

complete differentiation and maturation of pancreatic endocrine cells.

ENDOTHELIUM ROLE IN THE PANCREATIC NICHE

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

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

Embryonic endothelium and endoderm pre-patterning

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

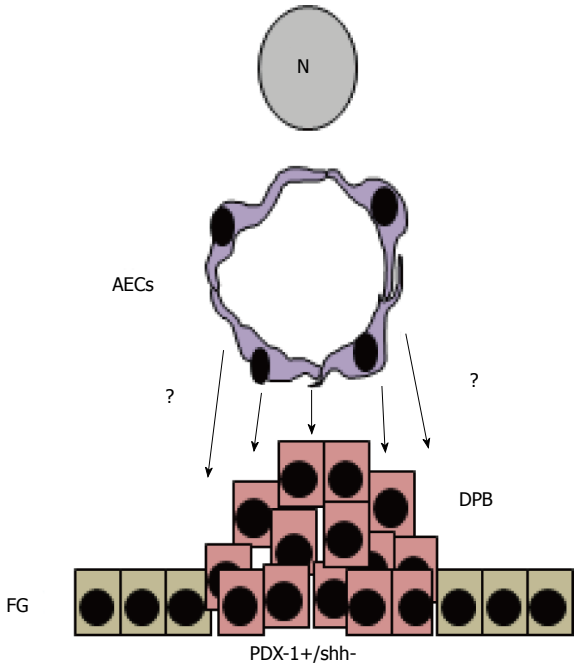

Figure 2 Endothelial-derived signals maintain expression of PDX-1 and promote pancreatic endocrine differentiation. Once the aorta is forming, aortic ECs (AECs) interact closely with FG/DE cells that maintain expression of PDX-1 and form the dorsal pancreatic bud (DPB). ECs: Endothelial cells; DE: Definitive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

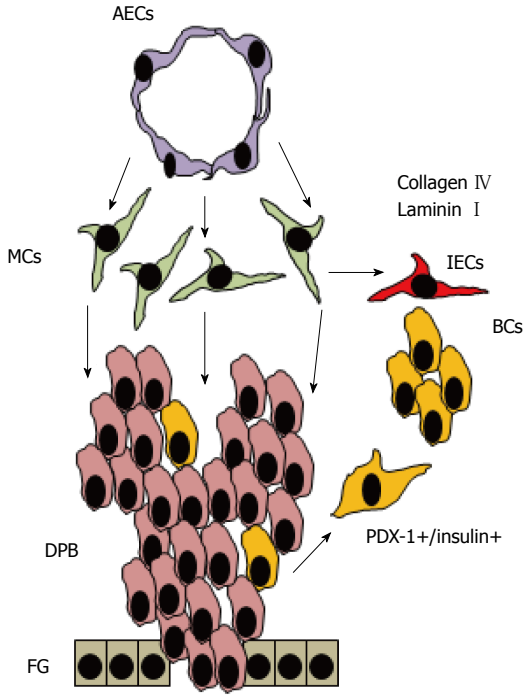

Figure 3 Endothelial-derived signals promote the survival of pancreatic mesenchyme which is essential for pancreas development. Mesenchymal cells (MCs) appear between AECs and the dorsal pancreatic bud (DPB) and promote proliferation and survival of differentiated cells. Immature beta cells (BCs) that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

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

Pancreatic specification induced by surrounding endothelium

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

Signals from pancreatic mesenchyme toward exocrine pancreas

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

ENDOTHELIUM ROLE IN PANCREATIC BETA-CELL MATURATION

Pancreatic endothelium during islet neogenesis

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

ENDOTHELIUM ROLE IN PANCREATIC BETA-CELL FUNCTION

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

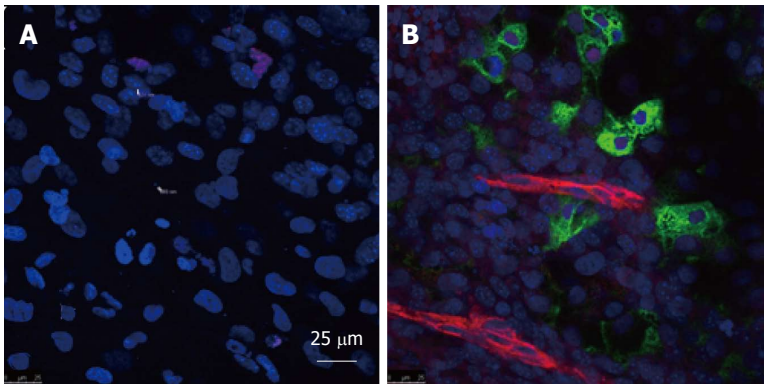

Figure 4 Analysis of blood vessels and insulin-producing cells in embryoid bodies obtained from human embryonic stem cell line H9. Human embryonic stem cells were cultured in suspension for 5 d to obtain embryoid bodies (EB). After attachment on coverslips for 24 h. Some EBs were cultured alone or together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

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

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

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

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

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

ENDOTHELIUM AND BETA-CELL PATHOGENESIS

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

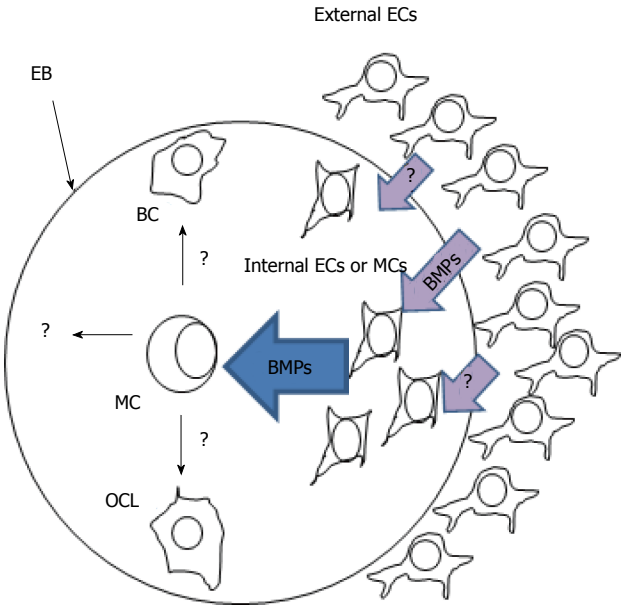

Figure 5 Diagram that explains the possible effects of external Endothelial cells toward *in vitro* beta-cell differentiation in human embryoid bodies. ?: Unknown factors, differentiation steps, or cell lineages; BMPs: Bone morphogenetic proteins; EC: Endothelial cells; EB: Embryoid bodies; BC: Beta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

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

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

CONCLUSION

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

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REVIEW

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

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Abstract

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

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

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

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INTRODUCTION

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

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

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

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

BIOMARKERS OF INFLAMMATION

Cytokines and acute phase proteins

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

CRP and PCT

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

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

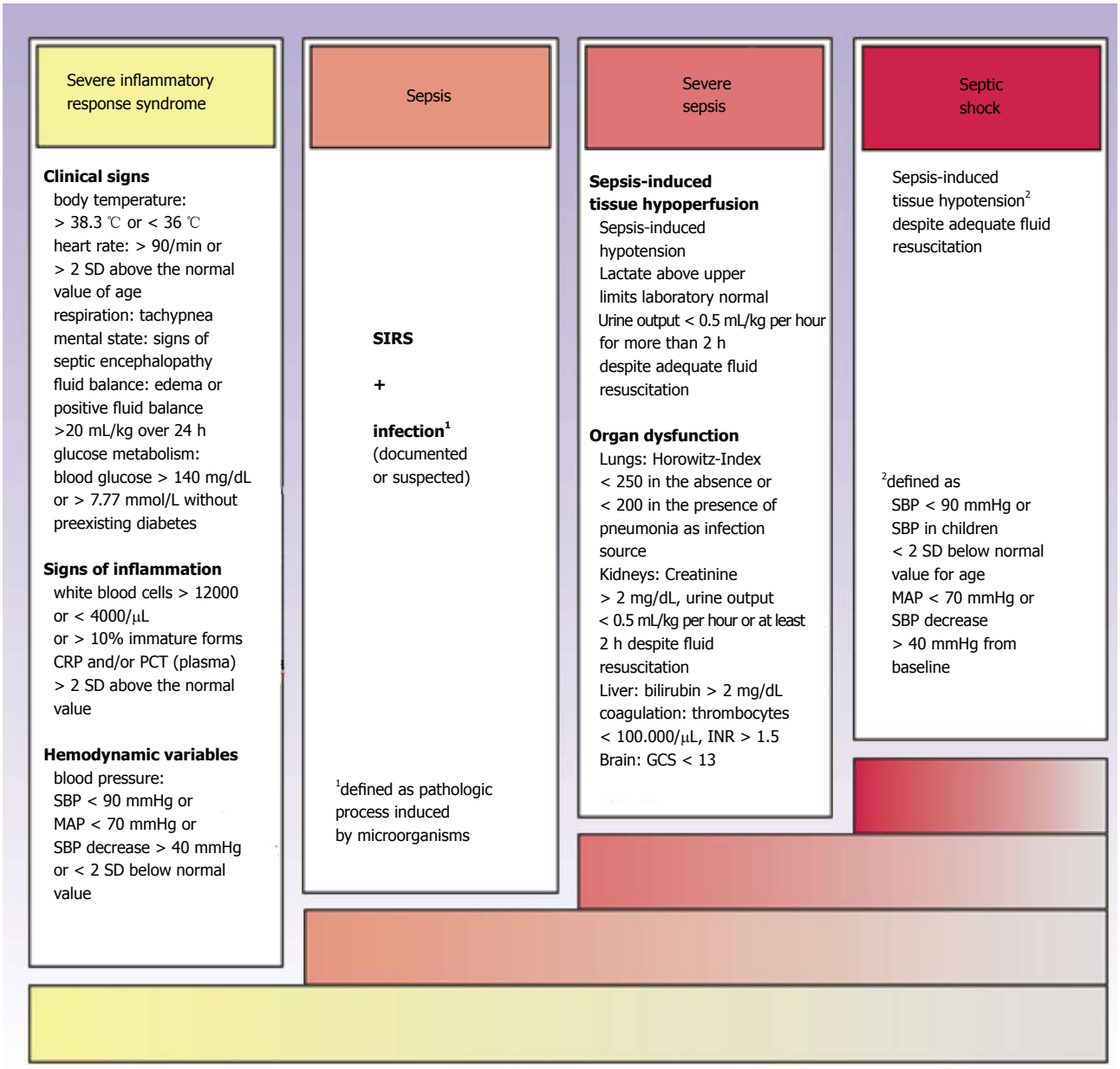

Figure 1 Diagnostic criteria of systemic inflammatory response syndrome, sepsis, severe sepsis and septic shock (modified from[5]). CRP: C-reactive protein; PCT: Procalcitonin; SIRS: Systemic inflammatory response syndrome.

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

IL-6

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

Pentraxin 3

The pentraxin superfamily comprises a wide range of

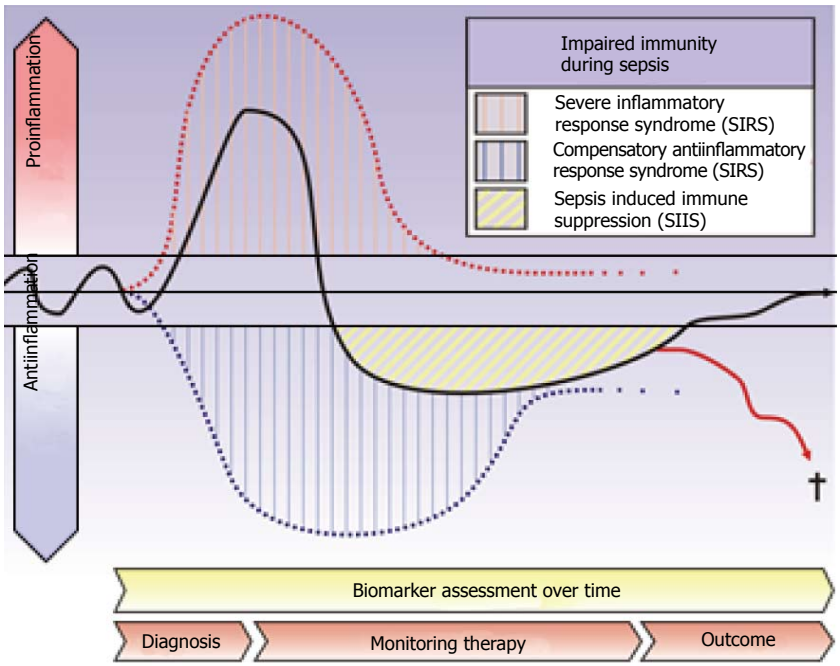

Figure 2 Simplified scheme of the impaired immunity during sepsis and the potential use of biomarkers: Initially, the body reacts to infectious stimuli with a proinflammatory immune response. Simultaneously, compensatory mechanisms are initiated to counteract the inflammatory process. The resulting net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

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

CELL-SURFACE RECEPTORS AND THEIR SOLUBLE FORMS

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

Soluble receptor for advanced glycation end products

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

Soluble triggering receptor expresses on myeloid cells-1

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

sCD14-ST

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

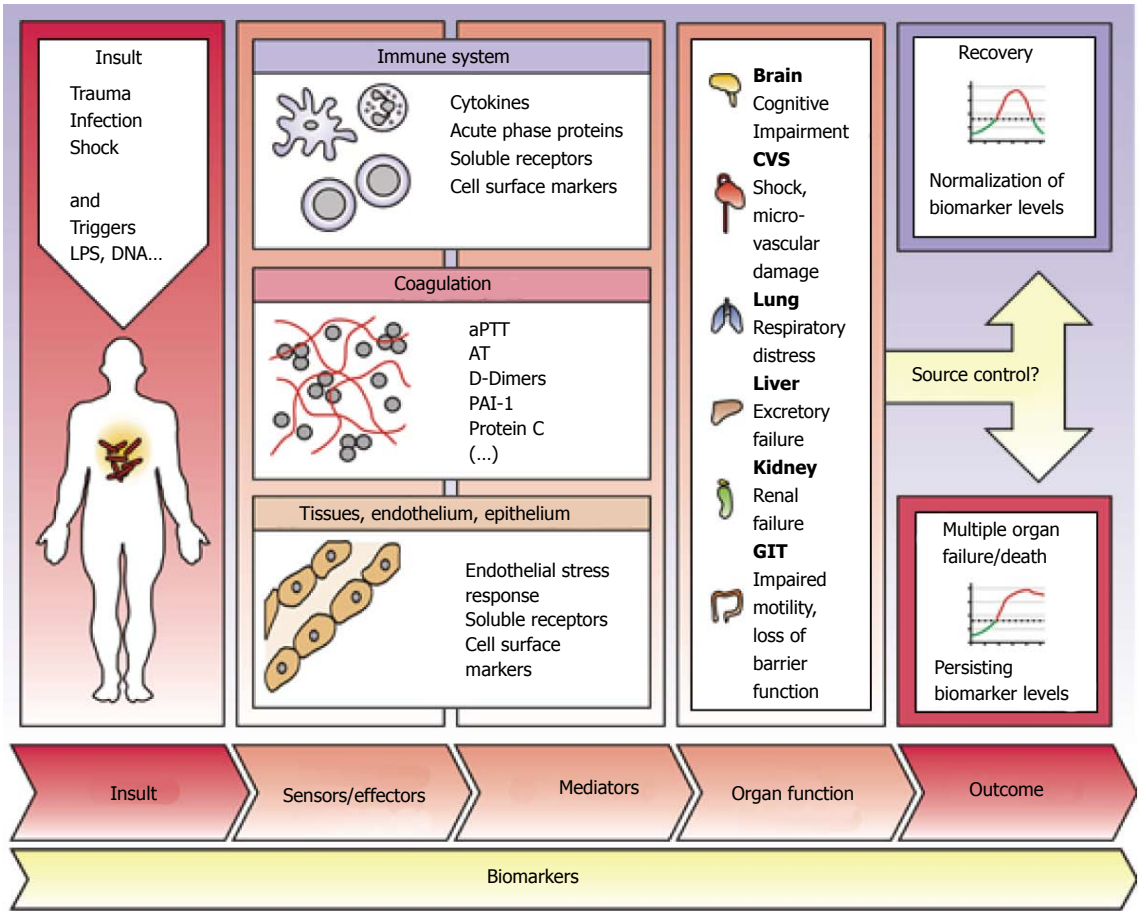

Figure 3 Use of biomarkers in sepsis: A wide range of biomarkers has been under intensive investigation to assist the clinician in diagnosis, outcome prediction and therapy guidance. LPS: Lipopolysaccharide; aPTT: Activated partial thromboplastin time; AT: Antithrombin; PAI-1: Plasminogen activator inhibitor-1;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

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

Soluble urokinase-type plasminogen activator receptor

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

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

Human leukocyte antigen-DR on circulating monocytes

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

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

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

BIOMARKERS OF TISSUE DYSFUNCTION AND ORGAN FAILURE

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

Heart type fatty acid binding protein

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

Troponin

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

Natriuretic peptides

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

Cytokeratin 18

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

Angiopoetin-1 und -2

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

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

Midregional-pro-adrenomedullin

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

Endocan

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

NON-LABORATORY BIOMARKERS

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

Body temperature patterns

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

Heart rate variability

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

Sidestream darkfield videomicroscopy

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

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

Clot-Lysis-Index

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

CONCLUSION

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

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

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

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

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

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

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

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REVIEW

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

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Abstract

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

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

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

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INTRODUCTION

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

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

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

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

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

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

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

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

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

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

NO AND RNS

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

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

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

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

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

NO, RNS AND INFLAMMATION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

NO, RNS AND AUTOIMMUNE DISEASE

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

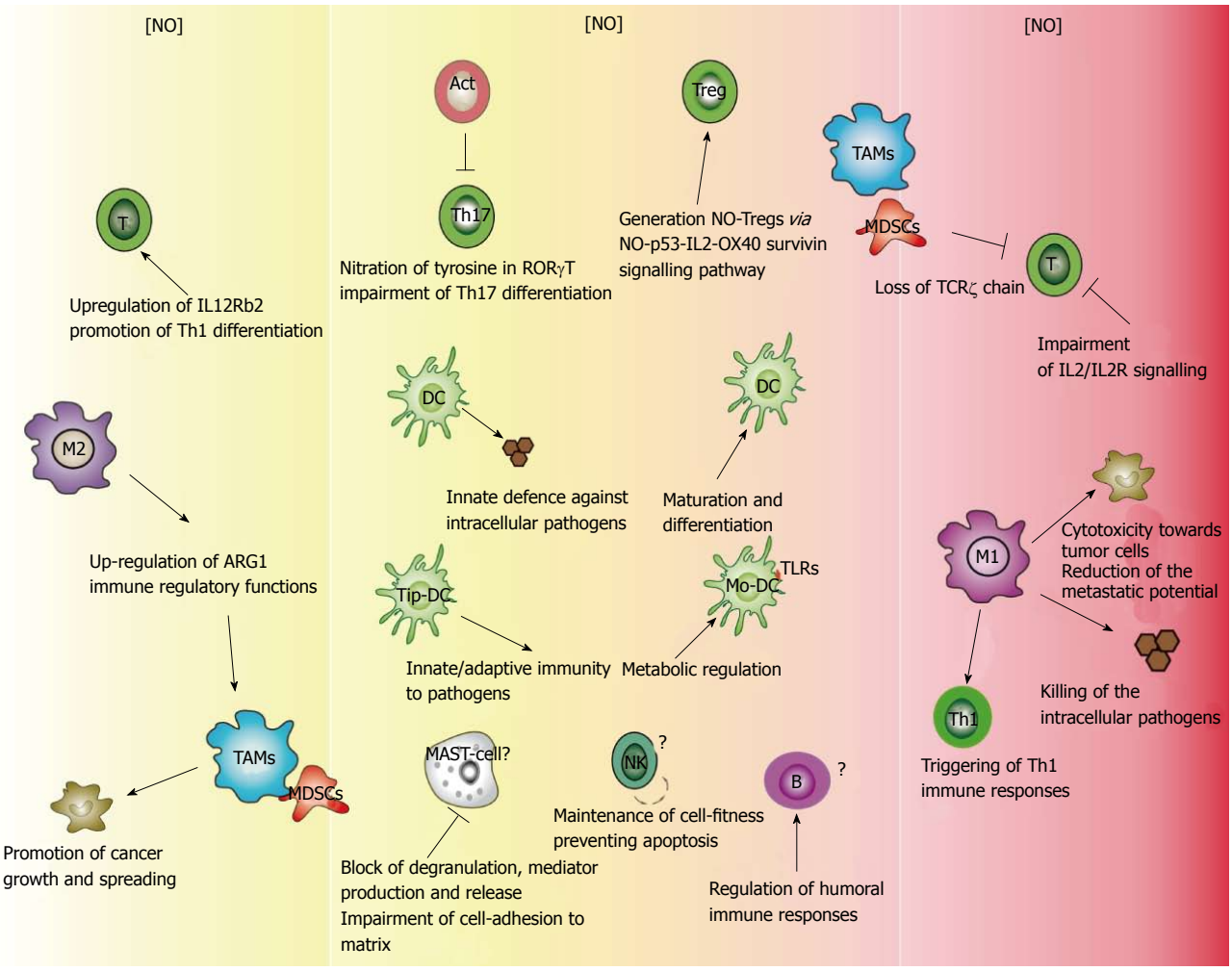

Figure 1 Distinctive effects of nitric oxide in immune cell subsets. Nitric oxide (NO) represents one of the key players involved in immunity and inflammation. The figure depicts the distinctive effects of NO within immune system, recapitulating its controversial behaviour. Low levels (left) could either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

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

NO, RNS IN DIABETES

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

Table 1 Nitric oxide in immune cells

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

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

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

NO, RNS AND NEUROINFLAMMATION

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

NO, RNS AND IBD

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

NO, RNS AND CANCER

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

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

Table 2 Nitric oxide and its effect in pathologies

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

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

CONCLUDING REMARKS

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

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

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

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

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

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

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

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REVIEW

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

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Abstract

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

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

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

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INTRODUCTION

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

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

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

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

OSAS AND INFLAMMATORY ACTIVATION

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

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

OSAS AND CRP

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

IS CRP PART OF THE LINK BETWEEN CVD AND OSAS?

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

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

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

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

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

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

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

CONFOUNDING FACTORS

Role of obesity

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

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

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

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

ROLE OF GENDER

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

EFFECT OF EXERCISE

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

Clinical implications

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

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

EFFECT OF POSITIVE AIRWAY PRESSURE THERAPY ON LEVELS OF CRP

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

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

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

CONCLUSION

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

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REVIEW

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

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Abstract

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

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

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

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INTRODUCTION

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

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

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

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

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

CANONICAL WNT PATHWAY

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

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

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

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

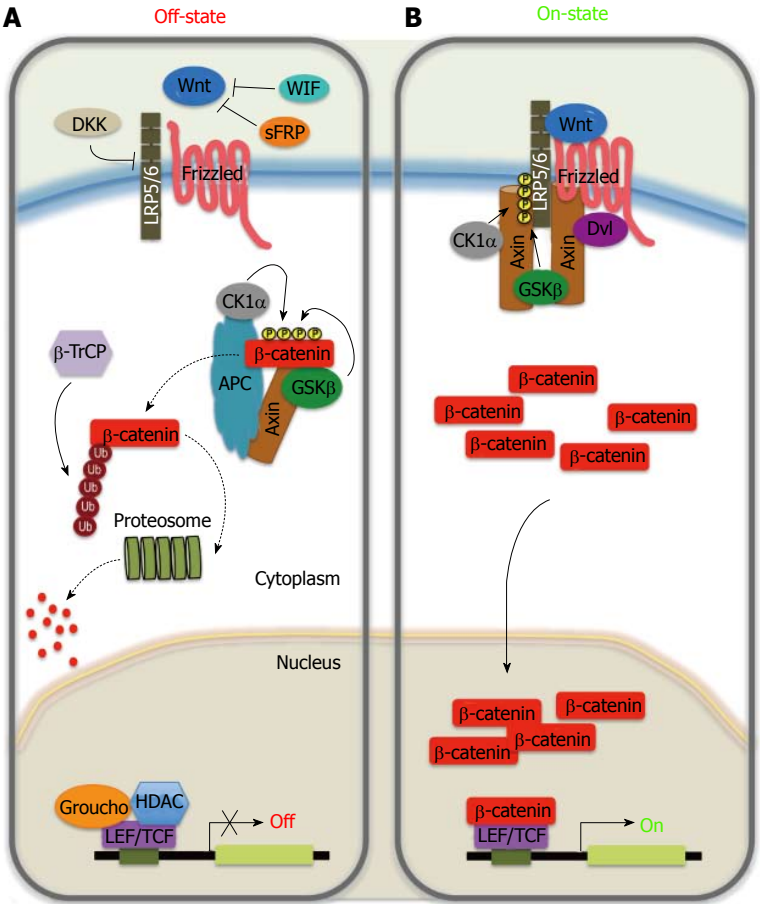

Figure 1 Wnt/β-catenin signalling pathway. A: In the absence of Wnt ligands, cytoplasmic β-catenin binds to the "destruction" complex composed by Axin, adenomatous polyposis coli (APC), the glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α). β-catenin is phosphorylated by the kinases of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

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

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

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

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

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

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

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

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

and RanBP3 have been implicated[35-41].

WNT/β-CATENIN PATHWAY IN GASTRIC CARCINOGENESIS

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

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

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

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

Gain of Wnt activator function in gastric cancer

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

adenocarcinoma formation by 1 year[72].

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

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

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

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

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

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

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

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

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

Loss of Wnt repressor function in gastric cancer

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

CONCLUSION

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

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

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REVIEW

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

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Abstract

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

Key words: Mood disorders; Ghrelin; Leptin

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

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INTRODUCTION

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

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

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

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

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

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

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

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

LEPTIN'S ROLE IN MOOD REGULATION

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

The HPA is a brain area that was systematically

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

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

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

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

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

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

GHRELIN'S ROLE IN MOOD REGULATION

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

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

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

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

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

MDD: Major depressive disorder.

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

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

DISCUSSION

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

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

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

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

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

CONCLUSION

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

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REVIEW

Asymmetric dimethylarginine, a biomarker of cardiovascular complications in diabetes mellitus

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Abstract

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

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

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

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INTRODUCTION

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

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

ENDOTHELIAL DYSFUNCTION, NITRIC OXIDE AND ADMA

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

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

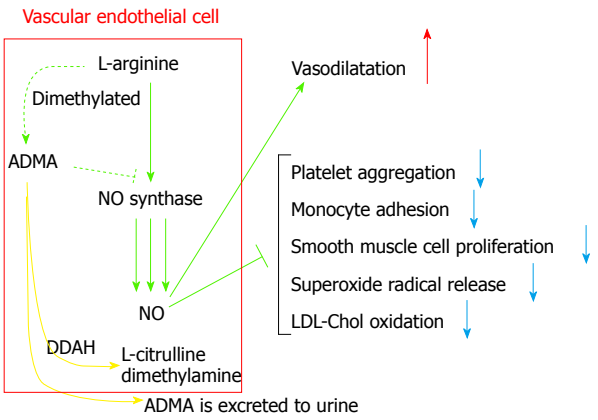

Figure 1 In non-diabetic patients, L-arginine by far outweighs asymmetric dimethylarginine, and active nitrogen oxide modulates vascular tone and structure. DDAH: Dimethylarginine dimethylaminohydrolase; ADMA: Asymmetric dimethylarginine; LDL: Low-density lipoprotein; NO: Nitrogen oxides.

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

PLEIOTROPIC EFFECTS OF ADMA

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

ADMA AND CV DISEASES

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

PHARMACOLOGICAL TREATMENT FOR CV DISEASES AND ADMA

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

Table 1 Characteristic of subjects

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

We enrolled 87 type 2 diabetic patients.

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

ADMA AND DM COMPLICATIONS

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

ADMA AND CAROTID INTIMA-MEDIA THICKNESS

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

OUR EXPERIENCE WITH ADMA

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

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

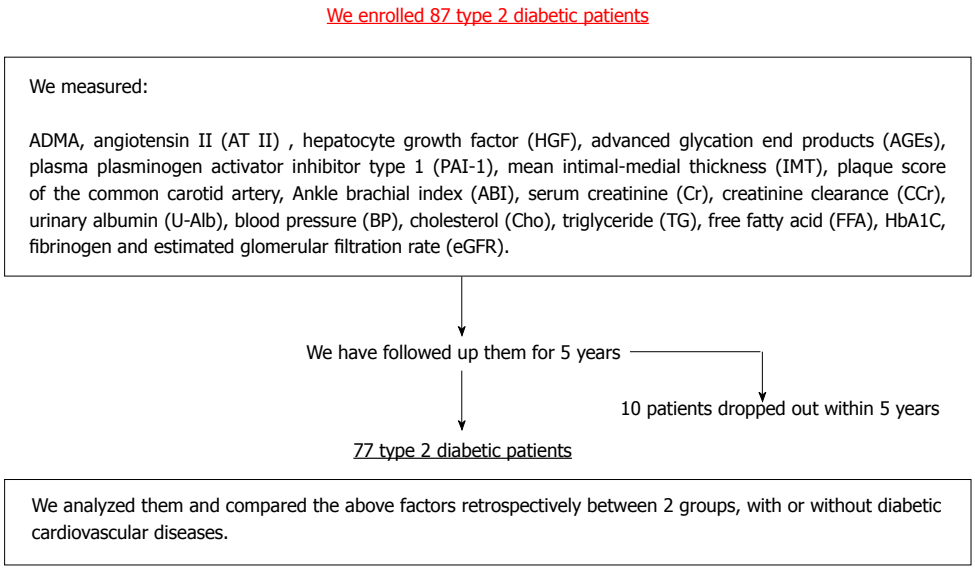

Figure 2 Protocols of our clinical research.

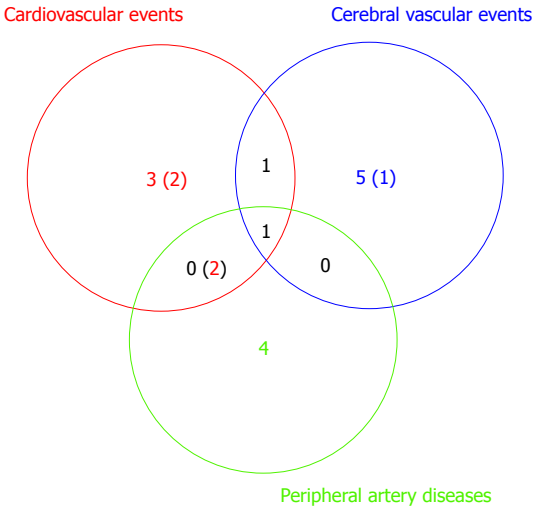

Figure 3 The result of diabetic cardiovascular disease that developed within five years. The numbers without parentheses mean new developments, and the numbers with parentheses mean recurrences.

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

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

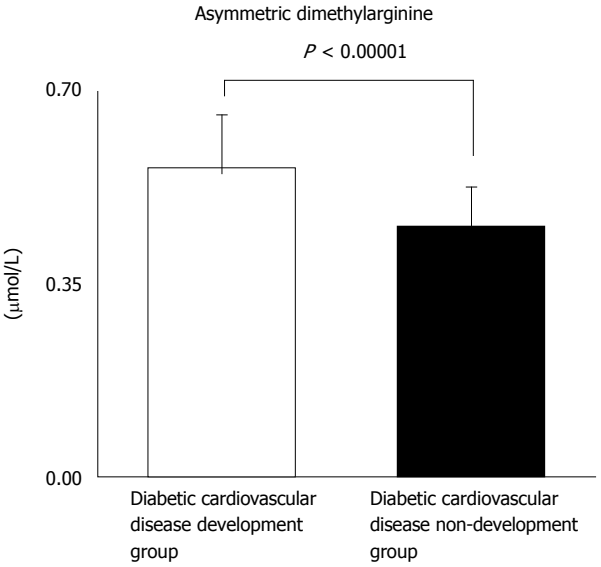

Figure 4 Asymmetric dimethylarginine was significantly higher in diabetic cardiovascular disease development group than in diabetic cardiovascular disease non-development group.

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

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

ADMA AND INCRETIN-BASED DRUGS

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

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

CONCLUSION

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

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MINIREVIEWS

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

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Abstract

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

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

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

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INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

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

CONCLUSION

IONM has some advantages over visual identification

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

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

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Abstract

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

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

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

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INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

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

FUTURE PERSPECTIVES IN *MEN1* GENETIC TEST

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

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

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

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

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

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

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

CONCLUSION

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

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

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

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

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Abstract

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

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

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

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INTRODUCTION

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

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

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

Epidemiology

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

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

Pathophysiology

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

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

CLINICAL PRESENTATION

Classification

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

Assessment of the foot

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

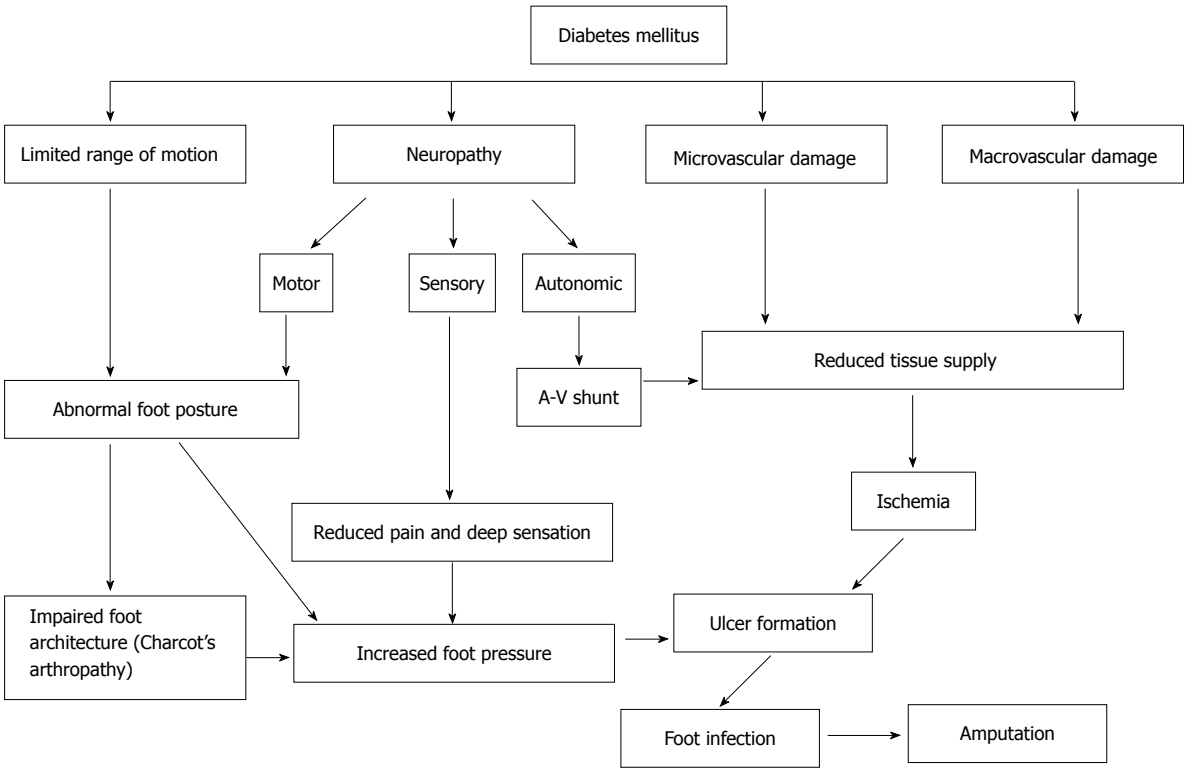

Figure 1 Diabetic foot pathogenesis.

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

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

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

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

Table 2 Physical therapy modalities potentially beneficial for wound healing

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

ESWT: Extracorporeal shock wave therapy.

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

PHYSICAL THERAPY AND REHABILITATION METHODS

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

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

Physical therapy modalities

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

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

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

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

Contraindications to ultrasound treatment include

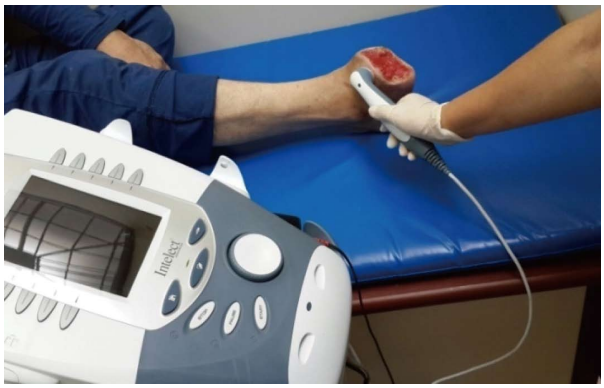

Figure 2 Ultrasound treatments administered following diabetic foot ulcer formation.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

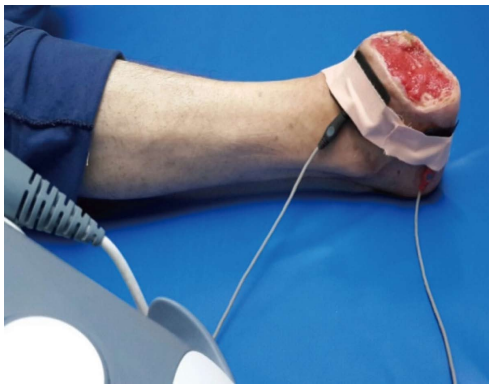

Figure 3 Galvanic current administered following diabetic foot ulcer formation.

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

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

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

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

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

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

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

CONCLUSION

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

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ORIGINAL ARTICLE

Clinical Trials Study

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

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Abstract

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

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

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

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

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

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

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

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Skeletal muscle tissues

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

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

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

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

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

Isolation and culture of MSCs

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

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

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

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

Animals and subcutaneous implants

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

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

Tissue processing and (immuno)histochemistry

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

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

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

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

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

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

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

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

Statistical analysis

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

RESULTS

Regeneration of ectopically implanted fresh human and mouse skeletal muscle tissue

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

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

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

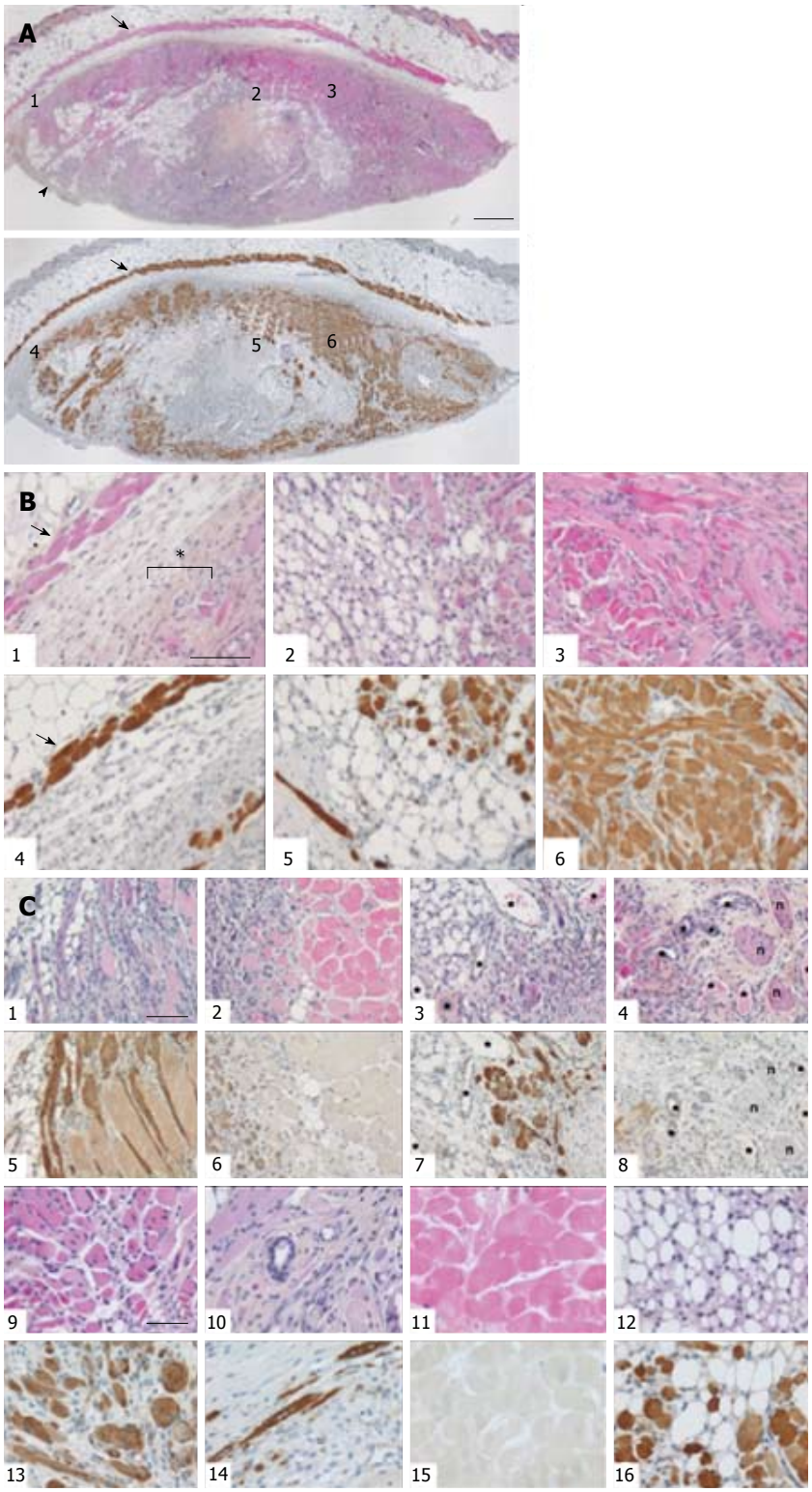

Figure 1 Histological analysis of subcutaneous murine muscle implants. A: Representative images of HPS-stained (upper panel) and desmin-stained (lower panel) sections of a cryopreserved mouse mince muscle implant at 30 d after implantation. From top to bottom are visible the skin layers (epidermis, dermis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

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

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

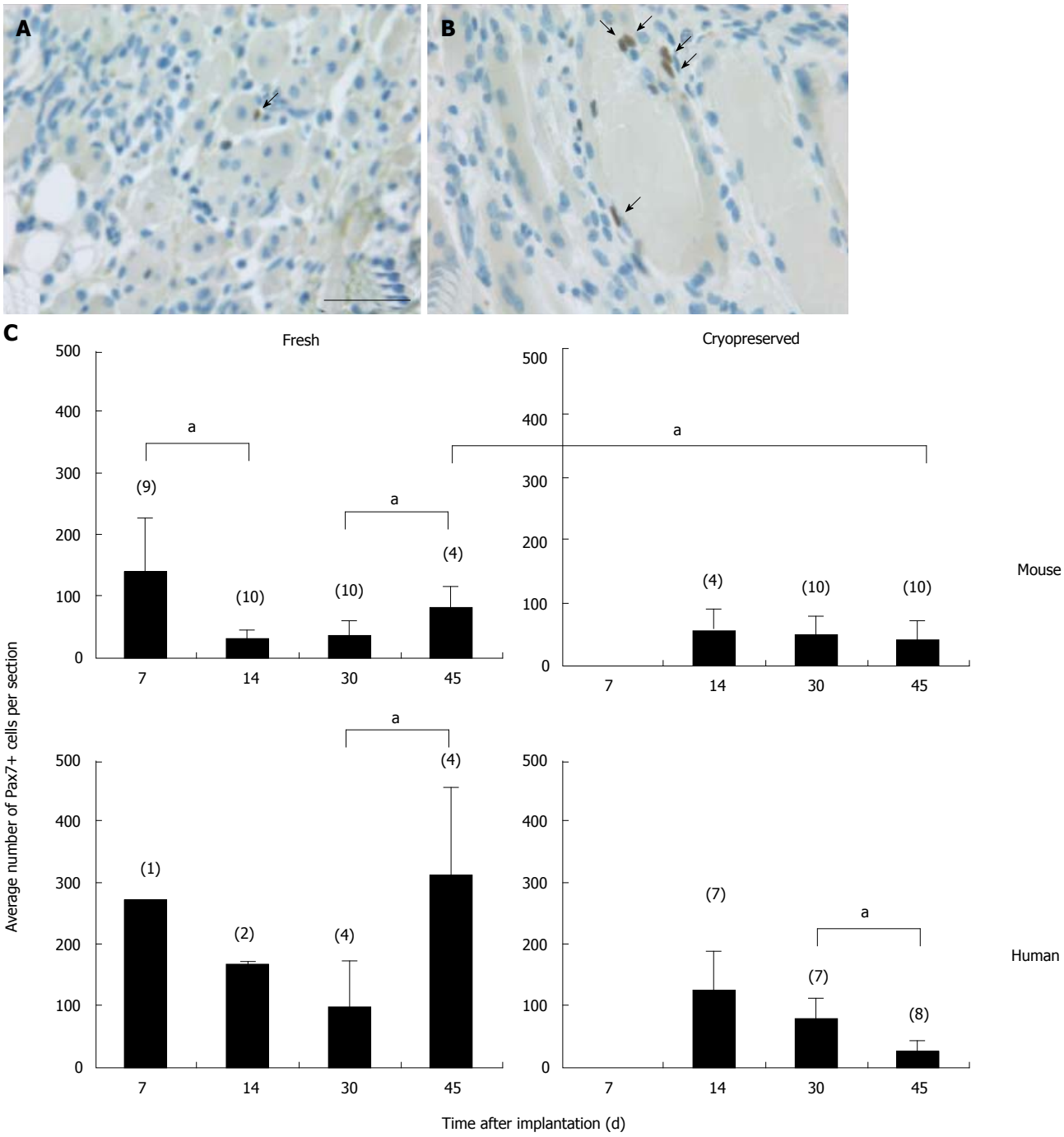

Figure 2 Pax7$^+$ cells in regenerating skeletal muscle implants. A, B: Examples of single (A and B) and pairs of (B) Pax7$^+$ cells (arrows) attached or positioned in close proximity to myofibers in a fresh mouse implant at 14 d after implantation. Scale bar is 50 μm; C: Pax7$^+$ cell counts in cross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

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

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

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

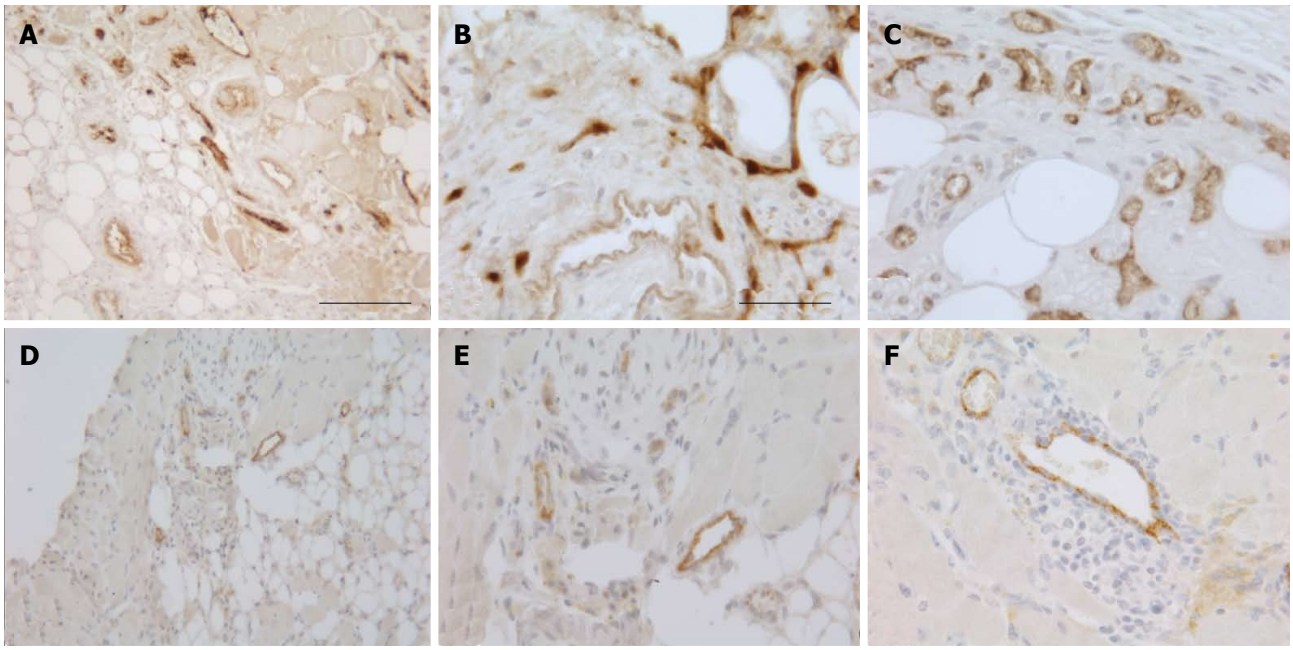

Figure 3 Vascularization of subcutaneous skeletal muscle implants. Photomicrographs of sections of fresh human (A-C) and mouse (D-F) minced implants at 30 d after transplantation stained for vWF (brown). Scale bar is 100 μm for (A, D) and 50 μm for (B, C, E, F).

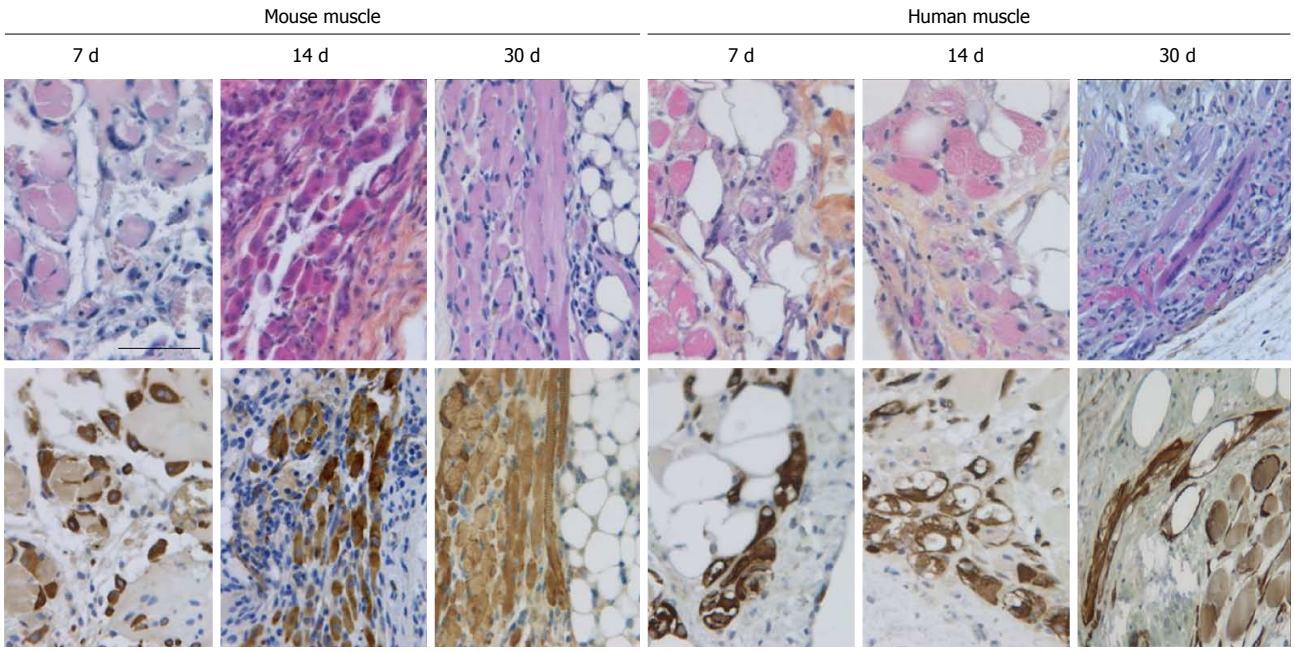

Figure 4 Myoregeneration of mouse and human minced cryopreserved muscle implants. Mouse and human fresh muscle implants were excised at different time points and stained with HPS (upper panels) or for desmin (lower panels). In the mouse implants progression of myoregeneration over time from myoblasts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

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

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

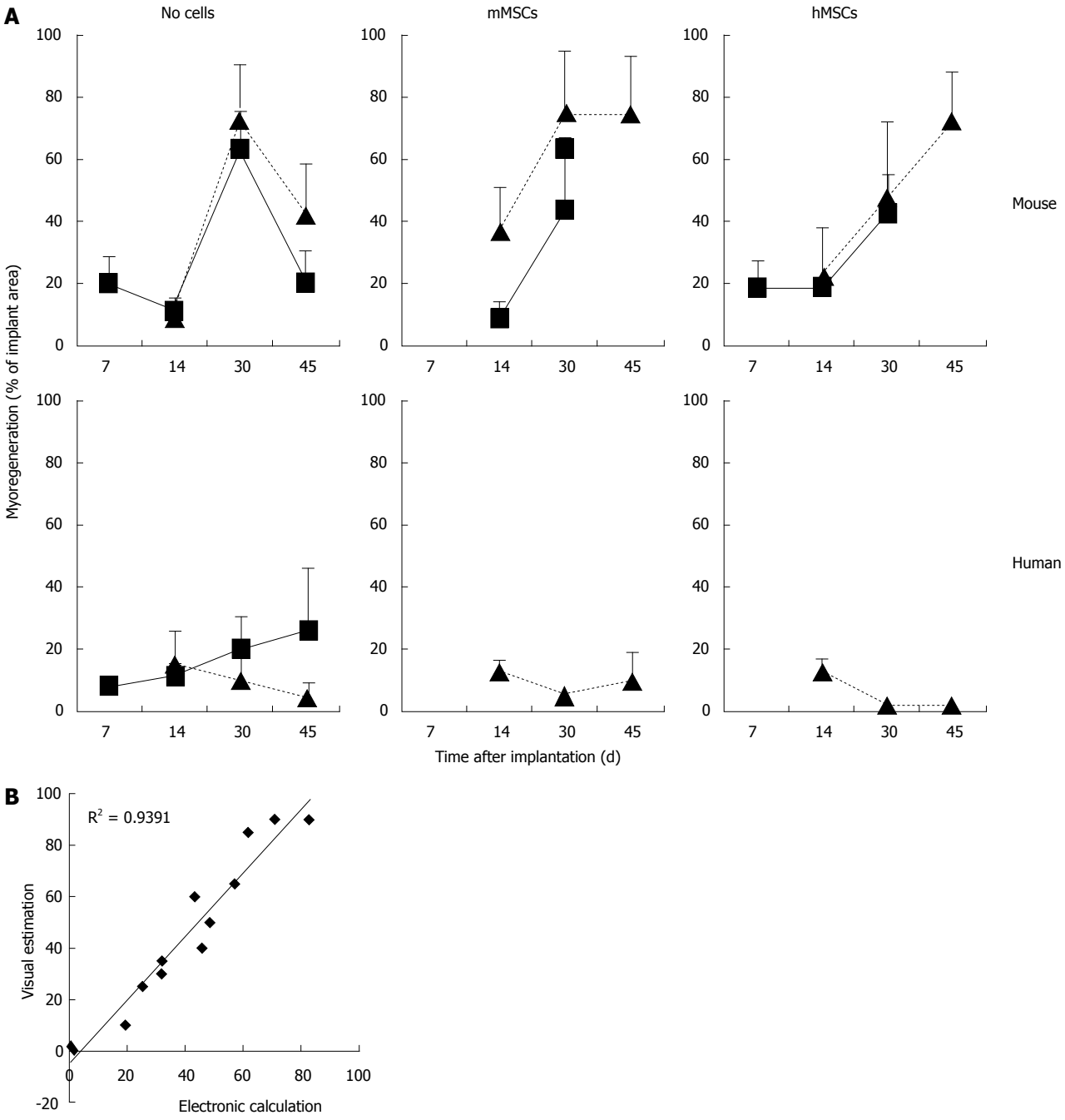

Figure 5 Myoregeneration kinetics of minced muscle implants without and with mesenchymal stem cells. A: The data represent visual estimates derived from microscopic examination of 2 or 3 desmin-stained sections through the center of fresh (square frame and solid line) and cryopreserved (triangle and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

Regeneration of cryopreserved muscle tissue

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

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

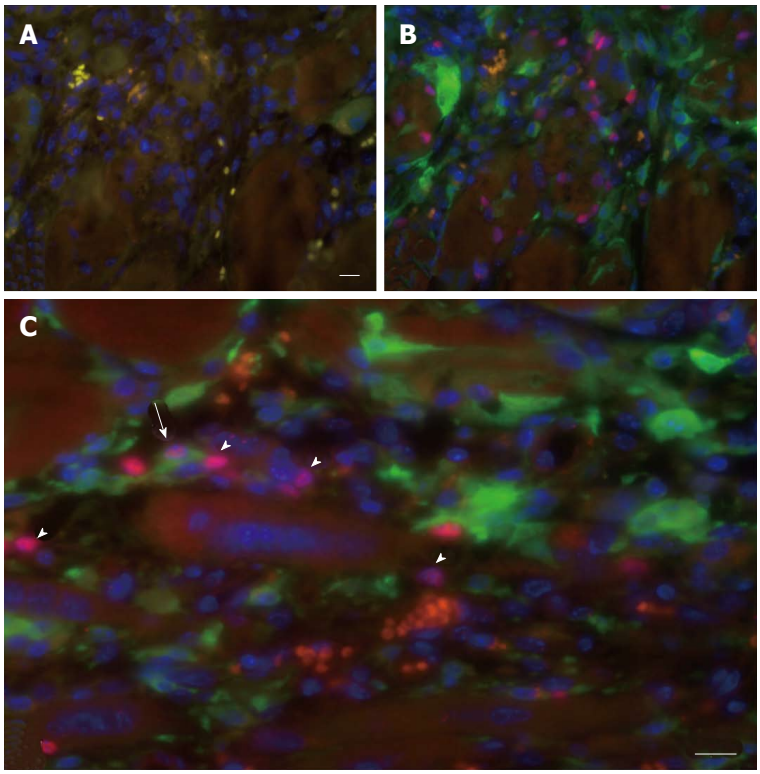

Figure 6 Contribution of host cells to myoregeneration in the muscle implant. Immunofluorescence analysis of a fresh mouse implant excised 7 d after implantation in eGFP transgenic recipients. Host cell contribution was evaluated in tissue sections stained with the karyophilic fluorochrome Hoechst 33342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

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

Contribution of host cells to myoregeneration in subcutaneous muscle implants

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

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

Effect of syngeneic or xenogeneic MSCs on myoregeneration in muscle implants

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

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

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

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

Myofiber calcification and cartilage formation in subcutaneous muscle implants

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

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

DISCUSSION

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

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

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

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

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

The origin of the cartilage, however, is more difficult

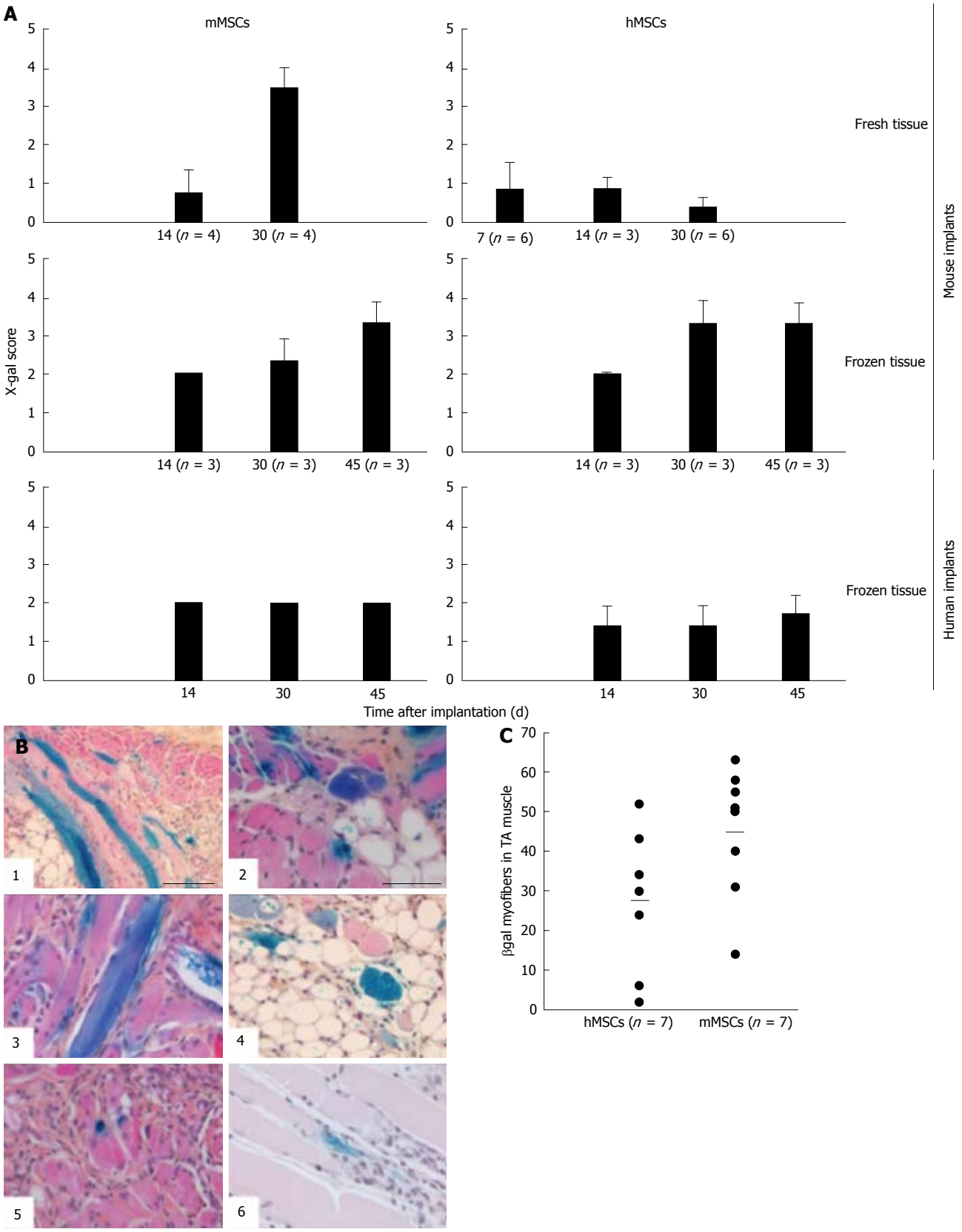

Figure 7 Contribution of mouse mesenchymal stem cells and human mesenchymal stem cells to myoregeneration in implants of fresh and cryopreserved mouse and human muscle minced. MSCs and their derivatives were identified by X-gal staining. A: For comparing implants we used an arbitrary score described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

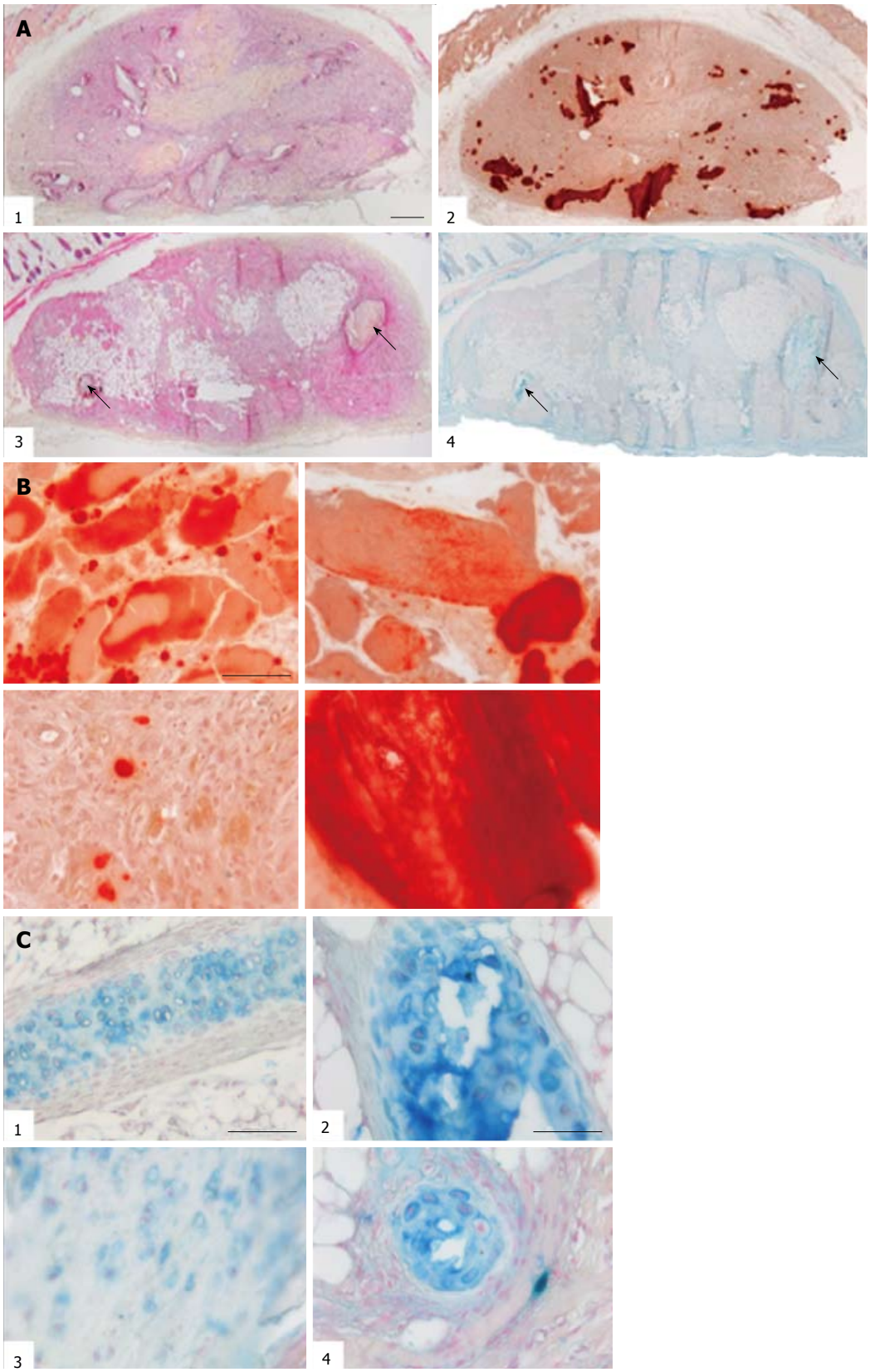

Figure 8 Myofiber calcification and cartilage formation in skeletal muscle implants. A: Human (1, 2) and murine (3, 4) muscle implants at 30 d after grafting stained with HPS (1, 3), Alizarin Red S for bone (2) or Alcian Blue for cartilage (4). Arrows indicate cartilage. Scale bar is 500 μm; B: Larger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

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

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

COMMENTS

Background

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

Research frontiers

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

Innovations and breakthroughs

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

Applications

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

Terminology

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

Peer-review

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

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