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Review

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Personalized Medicine

TBC update: personalized epigenetic management of diabetes

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The novel genome-wide assays of epigenetic marks have resulted in a greater understanding of how genetics and the environment interact in the development and inheritance of diabetes. Chronic hyperglycemia induces epigenetic changes in multiple organs, contributing to diabetic complications. Specific epigenetic-modifying compounds have been developed to erase these modifications, possibly slowing down the onset of diabetes-related complications. The current review is an update of the previously published paper, describing the most recent advances in the epigenetics of diabetes.

First draft submitted: 9 June 2017; Accepted for publication: 31 August 2017; Published online: 24 November 2017

Keywords: diabetes • epigenetic marks • epigenetics • personalized care • personalized management

Background

According to the latest survey, 415 million people worldwide (8.8% of adults) suffer from diabetes mellitus (DM), and the number will rise to 642 million by 2040 [1]. Epigenetic mechanisms regulate interactions between the genome and the environment factors, such as infections, nutritional changes and metabolic states. Disruption of epigenetic regulation contributes to the etiology of diabetes. Environmental exposures in the pre- and post-natal life are a large source of epigenetic variation with important role in diabetes development, specifically the lifestyle of Western-world, characterized by increased caloric intake and decreased energy expenditure. A comprehensive review of epigenetic management of diabetes has been published in 2015 [2]. In the present review, we discuss the latest published data on the issue.

Epigenetic modifications

Epigenetics studies the heritable changes in gene expression and phenotype that occur without alterations in the underlying DNA sequence.

Epigenetic modifications include: DNA methylation, histone modifications and noncoding RNAs, which determine chromatin remodeling and accessibility of transcriptional machinery to the genome or impact gene expression post-transcriptionally.

DNA methylation is a covalent addition of a methyl group to the cytosine residues in CpG dinucleotides. This reaction is catalyzed by DNA methyltransferases (DNMTs). DNMT3A and DNMT3B are *de novo* methyltransferases, while DNMT1 is involved in the maintenance of DNA methylation after replication. DNA methylation of promoter CpG islands is largely associated with gene repression, while their hypomethylation is associated with gene activation. In addition to transcriptional regulation, DNA methylation is critical for maintaining genome integrity, most of the genome being highly methylated. DNA methylation is not a stable epigenetic modification through life. Dynamic DNA methylation remodeling occurs during development and cell differentiation and it has been recently described during β-cell aging and reversal of high glucose (HG) insult on diabetic complications. Hydroxymethylation via 10–11 translocase (Tet) is the main mechanism for demethylation.

Histone modifications occur mainly by acetylation or methylation of the N-terminal tails of the histones. Histone modifications alter chromatin compaction and recruitment of transcriptional regulators, modifying gene expression. Histone acetylation occurs on lysine residue, leading quite rapidly to increased gene expression, while histone methylation is a more stable modification, leading to either gene activation or repression depending on which amino acid is modified (lysine or arginine) and extent of methylation (mono-, di- or tri-methylation). Thus, H3K9ac, H3K14ac, H4K5ac, H3K4me1/2/3 and H3K36me2/3 are representative transcriptionally active marks, whereas H3K9me3 and H3K27me3 are repressive ones. Many families of histone-modifying enzymes have been identified, including histone deacetylases (HDACs), acetyltransferases (HATs), methyltransferases (HMTs) and demethylases.

miRNAs are small noncoding RNAs (21–25 nucleotides length) that bind to the 3′-untranslated regions of target mRNA transcripts and disrupt their translation or lead to their degradation. miRNAs provide a rapid but reversible regulation of about 60% protein-coding genes, which allow the cell to adapt to the environmental changes.

Long noncoding RNAs (lncRNAs; >200 nucleotides length) impact gene expression by several mechanisms such as: the recruitment of epigenetic modifier proteins, transcription factors or control of mRNA degradation.

Epigenetic modifications in pancreatic development & maintaining β-cell identity

Data from genome-wide association studies point toward pancreatic β-cell dysfunction, as a key defect causing both types of diabetes.

A well-characterized transcription factor cascade controls the pancreatogenesis. There is evidence of epigenetic modifications that accompanies both, gene activation and silencing, essential for the differentiation process. Specific chromatin-modifying factors, like HDACs and the polycomb repressive complexes (PRCs) act sequentially and drive cellular differentiation. Two distinct PRCs, PRC1 and PRC2 are key regulators in coordinating gene silencing [3]. PRC2 suppresses transcription by catalyzing H3K27me3 (repressive modification). PRC1 subsequently recognizes H3K27me3 and facilitates chromatin compaction by histone 2A ubiquitylation, which in turn blocks recruitment of the H3K4 methyltransferase MLL1, an activator of gene expression. Deletion of the PRC2 component Ezh2 throughout the embryonic pancreas results in increased β-cell mass, while deletion of the PRC1 component Ring1b in multipotent progenitors but not in adult β cells results in re-expression of genes normally repressed in β cells. Lenoir *et al.* showed that class IIa HDACs control development of insulin-producing β cells and somatostatin-producing δ cells [4].

Following cellular differentiation, specific epigenetic modifications have central role in maintaining β-cell identity. Thus, maintenance of β-cell identity requires epigenetic repression of Arx, a transcription factor critical to α-cell fate. *Arx* is repressed in β cells by DNA methylation of the promoter [5].

Epigenetics controls also pancreatic endocrine cell function and glucose homeostasis. The insulin gene is epigenetically silenced in non-β cells by DNA methylation, while in β cells is dynamically regulated by epigenetic mechanisms in response to glucose levels. Thus, at low glucose levels, the transcription factor Pdx1 associates with HAT to decrease insulin transcription, while at HG levels Pdx1 facilitates recruitment of the HAT p300, which catalyzes H4 acetylation, and HMT Set7/9, which catalyzes H3K4me2, to create an active chromatin structure [6]. The HMT Set7/9 also promotes expression of other important glucose responsiveness genes such as *MafA*, *Slc2a2* and *Ins2* [7]. Depletion of Set7/9 in primary mouse islets resulted in decreased H3K4me2 of these targets, decreased gene expression and impaired glucose-stimulated insulin secretion. Interestingly, Set7/9 and Pdx1 also appear to directly regulate one another. *Setd7*, the gene encoding Set7/9, has an islet-specific enhancer occupied by Pdx1 enhancing transcriptional activity and β-cell glucose responsiveness [8].

Proliferation of pancreatic β-cell is an important mechanism for self-renewal and for adaptive expansion in response to insulin resistance (IR) from pregnancy or obesity. The cell-cycle inhibitor p16, encoded by the *Cdkn2a* gene, is a key mediator of the age-related decline in proliferative capacity. Recently, it has been shown that targeted *de novo* DNA methylation of the *Cdkn2a* gene decreased *Cdkn2a* expression and increased cell replication [9].

Several miRNAs regulate β-cell function. Thus, miR-375 is involved in glucose-dependent insulin expression through the regulation of PDK1 [10] while miR-9 regulates HDAc, SIRT-1 [11]. miR-24, miR-26, miR-148 and miR-182 increase insulin expression by downregulating insulin repressors, such as Bhlhe22 and Sox6 [12].

Global human β-cell transcriptome contains of >1000 lncRNAs, which are expressed in an islet-specific manner and exhibit dynamic regulation upon glucose changing or during differentiation [13]. It has been shown that lncRNAs act in concert with transcription factors. Thus, a specific lncRNA named PLUTO affects chromatin

structure and transcription of *PDX1*, and that both PLUTO and PDX1 are downregulated in islets from Type 2 diabetes mellitus (T2DM) donors, suggesting a potential role in the pathogenesis of human diabetes [13].

Epigenetic modifications in Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that involves T cell-mediated destruction of pancreatic β cells on the background of chronic β-cell inflammation.

T1DM results from a complex interaction between multiple genetic defects and environmental factors. More than sixty genetic polymorphisms have been associated with T1DM, mainly related to lymphocyte activation, immune response and cytokine signaling [14]. However, genetics explain only partially the hereditability of T1DM. The contribution of environmental factors to the diabetes etiopathogenesis is suggested by: seasonality patterns and geographic distribution of the diseases, as well as the associations with some environmental factors (viral infections, immunizations, early exposure to cow's milk, vitamin D deficiency).

The genome-wide studies showed that T1DM patients have an increase in global genomic DNA methylation in CD4+ T cells [15] and increase in the repressive mark, H3K9me2, at the promoters of several genes which are associated with the TGF-β, NF-κB and IL-6 signaling pathways [16]. Recently, it has been shown that CD4+T lymphocytes have also a dramatic reduction in global histone H3 acetylation, associated with glutamic acid decarboxylase autoantibodies titer, HbA_{1c} (reflects blood glucose control) and complications [17]. The low acetylation level was due to high expression of acetyltransferase CREBBP, as well as of HDAC1 and HDAC7. These repressive marks associated with decreases in the expression of immune response genes may contribute to the pathology of T1DM.

In T1DM, inflammatory and immune competent cells enter the islet and produce proinflammatory cytokines such as IL-1β, IL-12, TNF-α and If-γ; which impair β-cell function. During prolonged hyperglycemia, IL-1β decreases histone acetylation at the insulin promoter with subsequent decreased insulin expression, through loss of HAT p300 from the insulin promoter [18]. Thus, it is reasonable to target IL-1β to both prevent pancreatic β-cell loss and improve insulin expression.

A more recent genome-wide analyses showed that the levels of H3K9ac in blood monocytes were associated with the levels of HbA_{1c} in T1DM patients during Diabetes Control and Complications (DCCT) Trial and Epidemiology of Diabetes Interventions and Complications (EDIC) study. Genes with high H3K9ac levels were shown to be related to the NF-κB signaling pathway [19].

Epigenetic modifications in Type 2 diabetes mellitus

T2DM is characterized by hyperglycemia due to both impaired insulin secretion from pancreatic β cells and IR in insulin-sensitive tissues, including adipose tissue, skeletal muscle and liver. T2DM is a strongly heritable disease, but only approximately 15% of the observed heritability can be explained by known genetic variants. Data from genome-wide association studies identified >100 genetic variants associated with T2DM. Unfortunately, the odds ratios for individual risk alleles are generally <1.38 (the largest odds ratio for *TCF7L2*) [20]. This discrepancy may be explained by DM2 being a more heterogeneous disease than currently acknowledged and by epigenetic inheritance, whereby changes in gene regulation can be passed through generations. Strong evidence for the role of epigenetic factors in the pathogenesis of diabetes is sustained by the finding that intrauterine malnutrition leads to diabetes in adulthood (reviewed in [2]).

The transgenerational inheritance of famine

Children born to women who were pregnant during Dutch famine were small for gestational age and were more likely to develop diabetes later in life. Moreover, the trend was passed through generations. Hypomethylation of the imprinted *IGF2* gene has been described in this cohort [21].

In developed countries, fetal undernutrition related to placental insufficiency or restrictive diets is a common condition mimicking *in utero*-deprived famine cohort. Fetal undernutrition leads to intrauterine growth restriction, which predicts obesity and T2DM during adult life. Animal models of intrauterine growth restriction have been used for elucidating the mechanisms by which maternal nutritional during pregnancy affect the long-term metabolic health of their offspring (reviewed in [2]). A common finding of these models is the altered expression of key β-cell transcription factors, accompanied by epigenetic repressive modifications.

Maternal overnutrition is inherited to the next generations in the same way as maternal undernutrition, increasing the incidence of IR and diabetes in offspring. Exposure to a high fat diet (HFD) *in utero* affects glucose and

10.2217/pme-2017-0043

lipid metabolism of offspring through epigenetic modifications to adiponectin (decreased H3K9ac and increased H3K9me) and leptin genes (increased H4K20me) for several generations [22].

Evidence showed that exposure to maternal hyperglycemia during fetal growth is a risk factor for the development of T2DM in later life. The effects of intrauterine exposure may be confounded by inheritance of diabetes susceptibility alleles. In studies of discordant siblings for intrauterine exposure to diabetes, the risk was higher in those born after their mother developed diabetes than in those born before their mother developed diabetes [23]. However, the exact mechanism is largely unknown. Recently, it has been shown that hyperglycemia impairs differentiation of human embryonic stem cell (VAL3) into definitive endoderm (DE) via the modification of histone methylation. Increased repressive mark, H3K27me3, on the promoters of DE markers (*SOX17, FOXA2, CXCR4* and *EOMES*) after hyperglycemic treatment was associated with the downregulation of their expression [24]. Treatment with the H3K27me3-specific inhibitor, Adox, leads to the restoration of DE marker gene expression. A recent epigenome-wide association study showed that 48 differentially methylated CpG sites were associated with exposure to diabetes *in utero*, even after many years from exposure (average 13 years). Methylation status at some of these sites can impair insulin secretion, increase body weight and increase risk of T2DM [25].

Postnatal environmental exposures are a large source of epigenetic variation. It is generally accepted that diet, physical activity and BMI affect gene expression, while epigenetic regulation could be the mechanistic link between them. Several diet-induced metabolites including SAM, acetyl-CoA, NAD+ and ATP are cofactors for epigenetic enzymes, upregulating/downregulating transcription for maintaining homeostasis [26]. Thus, SIRT1 is a NAD+-dependent deacetylase and its activation depends on NAD+ bioavailability; DNMTs and HMTs use S-adenosylmethionine, while HATs utilize acetyl-CoA as an acetyl donor group.

Certain plant-derived extracts contain several bioactive molecules with HAT or HDAC inhibitors (HDACi) activity, like: anacardic acid (cashew nuts), garcinol (kokum fruit), curcumin (turmeric), lunasin (soya), sulforaphane (cruciferous), allyl mercaptan and diallyl disulfide (garlic) [26]. They are not as potent as synthetic inhibitors, but their abundance in the diet may have beneficial effect.

Exposure to pollutants, such as bisphenol A may also result in aberrant epigenetic modifications and impaired β-cell function. While it was already known that fetal exposure to bisphenol A in rodents led to altered glucose homeostasis, a recent study demonstrated that these effects correlated with changes in histone acetylation and methylation at the *Pdx1* locus [27].

Exercise also leads to modifications in the methylation status of gene regulatory regions in muscle and adipose tissue [28,29]; thus, epigenetic modifications may mediate IR that can result from reduced physical activity.

T2DM epigenetic signature in β cell

Prior studies using a candidate gene approach found increased DNA methylation in parallel with decreased expression of several β-cell genes in human pancreatic islets from patients with T2DM compare with nondiabetic ones. PGC-1α (encoded by *PPARGC1A*) is an important regulator of mitochondrial function, while *PPARGC1A* polymorphisms have been associated with increased risk of T2DM. Compared with control donors, in T2DM islets, the *PPARGC1A* promoter is hypermethylated and its expression reduced by 90% [30]. It has been also shown that DNA methylation of the *INS* promoter, at four specific sites, is increased in patients with T2DM compared with nondiabetic donors and it correlates negatively with *INS* gene expression in human pancreatic islets [31]. Pancreatic islets from patients with T2DM exhibited increased DNA methylation and decreased expression of PDX-1, and correlate with insulin secretion [32]. GLP-1 is secreted by gastrointestinal L-cells in response to meal intake and induces insulin secretion upon its binding to GLP1R in pancreatic β cells. T2DM patients have increased DNA methylation with reduced expression of GLP1R in pancreatic islets [33]. None of these studies could demonstrate that an abnormal methylation status of any single gene leads to the onset of diabetes, together they suggest that abnormal epigenetic regulation can contribute to the disease.

A more recent genome-wide DNA methylation analysis of human pancreatic islets from T2DM identified 102 differentially methylated and expressed genes compare with nondiabetic controls [34]. Although most of these genes had decreased methylation level compare with control, those individual CpG sites showing increased methylation by the candidate gene approach (mentioned above) showed concordant results. Moreover, 13 additional CpG sites annotated to the *INS* gene with increased DNA methylation in T2DM islets were identified [34].

An increased gene expression of HDAC7 in diabetic pancreatic islets has been also described in study mentioned above [34]. Recently, the upregulation of HDAC7 has been replicated and shown to be negatively correlated with insulin secretion from human islets [35]. HDAC7 impairs mitochondrial function, increases β-cell apoptosis and

increases Tcf7l2 expression. Moreover, the treatment of HDAC7-overexpressing β cells with MC1568, a selective class II HDACs inhibitor restored insulin secretion. These experiments support the development of an HDAC7-specific inhibitor as a therapeutic option for the treatment of T2DM [35].

T2DM epigenetic signature in peripheral blood cells

Genome-wide analysis in human peripheral white blood cells revealed low methylation level at specific sites, in young people that will later progressed to T2DM, compare with those who stayed healthy. As these DNA methylation variations increase individual susceptibility to T2DM, they might be considered early markers of T2DM [36]. Luttmer *et al.* showed similar results, people with T2DM or impaired glucose metabolism had DNA hypomethylation in peripheral white blood cells compared with normoglycemic individuals [37].

Another study investigating the global DNA methylation profile as a potential biomarker for the early detection of T2DM showed decreased global DNA methylation in the white blood cells of HFD monkeys compared with monkeys fed a standard diet. Moreover, low methylation level was inversely associated with body weight and plasma glucose levels. Thus, a healthy diet, reach in functional foods, may potentially reverse the methylation changes and prevent T2DM [38].

TCF7L2 is the susceptibility gene with the largest effect on T2DM risk. However, the mechanisms by which *TCF7L2* contributes to the disease are largely elusive. A recent methylation analysis of *TCF7L2* promoter in whole blood DNA extract showed that 16 CpGs sites out of the 22 CpGs analyzed (64%) showed significant differences in DNA methylation values between T2DM patients and controls. Moreover, methylation at four specific CpG sites in *TCF7L2* promoter was correlated with fasting plasma glucose levels [39].

The transcription factor NF-κB plays a central role in regulating inflammatory gene expression. Its effects are optimized by various coactivators including HATs, such as CREB-binding protein (CBP)/p300 and P/CAF. Evidence shows that HG conditions mimicking diabetes increase recruitment of the CPB/p300 and p/CAF, leading to increased histone acetylation at the *COX*-2 and *TNF*-α inflammatory gene promoters, with a corresponding increase in gene expression. The data have been confirmed *in vivo,* by demonstrating a similar pattern of increased histone acetylation (H3K9ac, H3K14ac) at the inflammatory gene promoters in monocytes from both T1DM and T2DM patients relative to nondiabetic [40]. The same group analyzed changes in histone methylation patterns in monocytes cultured under normal versus diabetic conditions [41]. The study demonstrated dynamic changes in both the H3K4me2-activation mark and H3K9me2-repressive mark in cultured monocytes treated with HG, and relevance to human diabetes was demonstrated by noting similar changes in monocytes from diabetic patients [41].

It has been shown that the histone H3-lysine 4 methyltransferase, SET7/9, is a coactivator of NF-κB. Gene silencing of SET7/9 in monocytes inhibited TNF α-induced inflammatory genes and histone H3-lysine 4 methylation on these promoters, as well as monocyte adhesion [42]. Similarly, a role for SET7/9 in regulating NF-κB-induced inflammatory gene expression in response to HG was shown in endothelial cells [43].

Recent findings suggest that MAP4K4 downregulation and subsequent IL-6 overproduction in human T cells play important roles in the pathogenesis of nonobese T2DM [44]. Analysis of common SNPs in the *MAP4K4* locus of 1769 DNA samples from prediabetic Europeans identified two SNPs in the *MAP4K4* locus to be associated with increased glucose levels in patients, while two other SNPs were associated with reduced insulin release only in lean subjects [45]. Consistently, MAP4K4 expression levels were decreased and IL-6 production was increased in T cells from drug-naive, nonobese T2DM patients [44]. Moreover, the methylation frequencies of the *MAP4K4* promoter were increased in T2DM patients and correlated with glucose tolerance tests, independently of BMI, smoking, gender or age. Demethylation treatment increased MAP4K4 expression levels and reduced IL-6 production in T2DM T cells [44]. This report identifies *MAP4K4* methylation in T cells as a potential biomarker for non-obese T2DM.

T2DM patients have lower circulating levels of miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR- 223, miR-320 and miR-486 compare with controls subjects. Moreover, some of them have changed before the overt disease and may serve as biomarkers for the early detection of T2DM [46]. More recently, circulating lncRNAs have been proposed as diagnostic biomarkers for prediabetes and T2DM [47].

Diabetes epigenetic signature in insulin-sensitive tissues

PPARγ is a key transcription factor in adipocyte differentiation and function. Increased methylation of the PPARγ promoter in visceral adipose tissues, concomitant with its decreased expression was observed in obese diabetic mouse [48]. The thiazolidinedione antidiabetic drugs are potent activators of PPARγ. A recent genome-wide

10.2217/pme-2017-0043

methylation analysis showed differential DNA methylation in adipose tissue from subjects with T2DM compared with controls, in >7000 genes including *PPARG*, *KCNQ1, TCF7L2* and *IRS1* [49].

Prior studies have shown that increased fat accumulation, mainly as visceral fat, alter adipokines secretion (increase TNFα, IL-6, leptin and/or decrease adiponectin) and results in IR and T2DM. Epigenetic regulation has been involved in the adipokines expression. Decreased adiponectin gene expression in 3T3-L1 adipocytes with IR was accompanied by decreased H3K9ac at the promoter gene [50].

miRNAs have proved to be negative regulators of insulin sensitivity and glucose homeostasis. The expression of miRNAs miR-103, miR-107 [51], miR-143 [52], miR-181 [53], miR-29 [54] and miR-802 [55] has been shown to be upregulated in livers of obese rodent models. Data have been recently replicated in rodent models by Kornfeld *et al.* [55], but also in the liver of patients with IR and hepatic steatosis (miR-103, miR-107), obese patients (miR-802 [55]) and T2DM patients (miR-801 [53]). Their expression levels positively correlated with the subjects' homeostatic model assessment (HOMA) index (for miR-103, miR-107) or HOMA index in mice (miR-143). The silencing of these miRNAs increased insulin sensitivity in liver (miR-143, miR-181, miR-802) or in the liver and adipose tissue (miR-103, miR-107), while their overexpression induces hepatic IR and impaired glucose tolerance. The target genes of these miRNAs have been identified, such as *Caveolin-1* (miR-103/107), *ORP8* (miR-143), *SIRT1* (miR-181), *TCF2* (miR-802) and *Akt* (miR-29).

A recent analysis in a large community-based cohort showed that 16 plasma-circulating miRNAs were associated with markers of IR, with an emphasis on miR-122, which was associated with IR (as measured by insulin and HOMA-IR), independent of age, sex and BMI [56]. However, miR-122 have been just slightly increased in the liver of obese mice, in the study of Kornfeld *et al.* [55]. The difference in the magnitude effect size could be explained by species differences or tissue specificity (liver vs plasma).

These findings sustain the potential therapeutic role of targeting these miRNAs in insulin-resistant DM2 patients.

A specific set of lncRNAs was upregulated during adipogenesis and was induced by the proadipogenic transcription factors CEBPA and PPARγ [57].

Many epigenetic studies in diabetes have not yet been replicated or the magnitude effect sizes are different. Few reasons could explain that, like: epigenetic regulation is tissue specific and it is crucial to collect and investigate high-quality tissues or cells that have an important role in the pathogenesis of T2DM; epigenetic regulations can be influenced by environmental factors such as age, obesity, physical activity and diet; the technology to investigate epigenetic changes has changed quickly in last few years, etc.

Epigenetic modifications in diabetes complications

Both diabetes types are associated with an increased risk of complications and hyperglycemia has been shown to be the major risk factor. Hyperglycemia can lead to the activation of several cellular pathways, including enhanced oxidative stress, activation of proinflammatory NF-κB, activation of PKC and TGF-β-MAPK signaling pathways, and increased formation of advanced glycation end products (AGEs).

Diabetic nephropathy (DN) is one of the major diabetic complications, characterized mainly by proteinuria and glomerular dysfunction.

Prior studies demonstrated that early characteristic features of DN include increased production of vasoactive factors such as endothelin 1 and increased synthesis of extracellular matrix proteins such as fibronectin (FN), type I and IV collagens, and laminin. Induction of profibrotic TGF-β1 in renal cells by diverse mediators such as HG, AGEs or angiotensin II promotes fibrosis and progression of DN.

TGF-β1 decreased H3K9me (repressive mark) and increased H3K4me (active mark) at fibrotic gene promoters (*CTGF, collagen-α1* and *PAI-1*), and induced the expression of a H3K4-methyl transferase SET7/9, in rat mesangial cells. Furthermore, a TGF-β1 antibody prevented similar HG-induced changes in mesangial cells [58] (Table 1). Moreover, overexpression of HATs CBP or p300, significantly enhanced TGF-β1-induced *PAI-1* in rat mesangial cells and in glomeruli from diabetic mice, while TGF-β1 treatment led to increased H3K9/14Ac and p300/CBP occupancies at the *PAI-1* [59].

In recent years, several other kidney-specific histone modifications have been proven on diabetic animal models. Chen *et al.* [60] focused on MCP-1, a proinflammatory cytokine that plays an important role in nephropathy progression. They showed that endoplasmic reticulum stress triggers MCP-1 expression through SET7/9-induced histone methylation in the kidneys of db/db mice. Thus, the increased active chromatin mark (H3K4me1) at *MCP-1* promoters has been accompanied by the upregulation of the MCP-1 expression. Other group focused on *OPN*, which is another gene upregulated in kidneys from diabetic mouse models as well as humans with DN,

Table 1. Epigenetic modifications in diabetic complication.

Target	Epigenetic findings in diabetic nephropathy	Ref.
Histone modifications		
CTGF, collagen-α1, and *PAI-1*	Decreased H3K9me and increased H3K4me in rat mesangial cells	[58]
MCP-1	Increased H3K4me1 in kidney of diabetic mice	[60]
Opn, Txnip	Increased H3K9ac, H3K4me1, H3K4me3; and decreased in H3K27me3 in kidney of diabetic mice	[61,62]
PAI-1 and RAGE	Increased H3K9/14Ac and decreased H3K9me2, H3K9me3 and H3K27me3 in the renal glomeruli of diabetic mice	[63]
DNA methylation		
TXNIP	Hypomethylation of the promoter in mouse mesangial cells, human podocytes, human serum	[65,66]
COLIVA1, RUNX3	Hypomethylation in human renal tubule	[67]
SMAD6, RARB	Hypermethylation in human renal tubule	[67]
Claudin-1	Deacetylation of histone H3 and H4 and subsequent cytosine hypermethylation in podocytes	[68]
Agt, Abcc4, Cyp4a10, Glut5, and Met	Hypomethylation in mice proximal tubule cells	[69]
Kif20b, Cldn18 and Slco1a1	Hypermethylation in mice proximal tubule cells	[69]
miRNAs		
TGF-β1, Col 1α2 and Col4α1	Increased miR-192, miR-200b and miR-200c in the glomeruli of diabetic mice and in mouse mesangial cells	[70,71]
Spry1	Increased miR-29c in kidney endothelial cells and podocytes	[73]
E-cadherin	Decreased miR-192 in a proximal tubular cell line and in human kidney biopsies	[74]
TSP-1	Increased miR-320c in urinary exosomes from DN patients	[75]
Epigenetic findings in diabetic retinopathy		
Histone modifications		
Sod2	Increased H4K20me3, H3K9ac and decreased H3K4 at promoter/enhancer in retina and RECs	[78,79]
MMP-9	Decreased H3K9me2 and increased H3K9ac at promoter in retina and RECs	[80]
Cox2	Decreased H3K9me3 and increased H3K9ac in RECs	[83]
Gulch	Decreased H3K4me1, H3K4me3 and increased H3K4me2 in the retina and RECs	[84]
Keap1	Increased H3K4me1 at promoter in retina and RECs	[85]
DNA methylation		
MMP-9	Hypomethylation of the promoter in retina and RECs	[81]
Polg1	Hypermethylation of the promoter in retina and RECs	[129]
miRNAs		
NF-κB	Increased miR-146, miR-155, miR-132, miR-21 in RECs	[88]
VEGF	Increased miR17–5p, miR-18a, miR-20a, miR-21, miR-31 and miR-133 in retina and RECs	[88]
p53	Increased miRNA-34 in retina and RECs	[88]
Oxr1	Increased miR-200b in retina	[89]
PACT/RAX	Increased miR-29b in retina	[90]
FN	Decreased miRNA-146a in retina and RECs	[130]
SIRT1	Increased miR-23b-3p and miR-195 in human RECs	[87,92]
Epigenetic changes in macrovascular complications		
ET-1, VEGF, FN	Histone acetylation in human endothelial cells	[131]
Hsp60	Increased miR-1 and miR-206 in cardiomyocytes	[94]

DN: Diabetic nephropathy; REC: Retinal endothelial cells.

10.2217/pme-2017-0043

and suggested to play an important role in the pathogenesis of DN. They demonstrated that the upregulation of the *Opn* gene in kidneys of diabetic mice was associated with increased active marks H3K9ac, H3K4me1 and H3K4me3 levels, and decrease in repressive mark H3K27me3 in the promoter region of the *Opn* gene [61]. The same group showed a similar histone modifications pattern at the gene promoter of *Txnip,* associated with its increased expression in diabetic mice [62]. Reddy *et al.* examined the effect of losartan, an angiotensin II type 1 receptor blocker, on epigenetics in diabetic mice [63]. Prior studies showed that angiotensinogen II signaling plays a critical role in the pathogenesis of DN, mainly by increasing the production of TGF-β1 and AGEs. Clinical studies confirmed that ARBs can slow down the progression of DN. They found that the elevated glomerular fractions of the *MCP-1, PAI-1* and *RAGE* genes in diabetic mice were associated with increased active mark H3K9/14Ac and decreased repressive marks H3K9me2, H3K9me3 and H3K27me3 at the *PAI-1* and *RAGE* gene [63]. They showed that several HATs, HDACs and HMTs were upregulated in the glomerular fraction of diabetic mice and were diminished by ARBs.

These results indicated that histone modifications play a critical role in DN and that targeting histone modification seems a promising strategy against DN. Indeed, two inhibitors of methyltransferase EZH2 (3-DZNeP, GSK126), which trimethylates H3K27, were shown to ameliorate renal fibrosis induced by unilateral ureteral obstruction [64].

Prior studies have shown that high glucose augments the expression of *TXNIP* promoter, an endogenous inhibitor of the ubiquitous oxidoreductase thioredoxin, promoting oxidative stress and diabetic complications. Recently, it has been shown that TXNIP has critical role in pathogenesis of DN. Systemic knockout of the *TXNIP* gene ameliorates streptozotocin-induced DN [65]. Interestingly, a recent DNA methylome analysis of T1DM participants in the DCCT/EDIC Study showed a hypomethylation of the *TXNIP* promoter, associated with diabetic complications, that persisted over 16 years [66]. Persistent hypomethylation in this gene may render the patients vulnerable to DN.

DNA methylation analysis in microdissected tubules of kidneys of diabetic kidney disease patients showed differential methylation changes concordant with differences in gene expression in the TGFβ pathway (especially in *TGFBR3, SMAD3, SMAD6*), a well-known regulator of kidney fibrosis development [67].

Several additional changes in DNA methylation profiling have been described in diabetic-kidney disease animal models. Hasegawa *et al.* reported that Sirt1 has a protective effect in renal damage. Sirt1 regulate expression of the tight junction protein Claudin-1 through deacetylation of histones H3 and H4. Subsequently *Claudin-1* is hypermethylated by recruited DNMT1. Moreover, *Sirt1* transgenic and knockout mice showed inhibition and worsening of the glomerular damages occurring in diabetes, while in diabetic patients, Sirt1 and Claudin-1 levels were correlated with proteinuria level [68].

Another report on the DNA methylation profile of purified proximal tubules of diabetic mice kidneys, showed several differentially methylated sites and highlighted the hypomethylation of the *Agt* gene, since the Agt protein, or angiotensinogen, is closely related to the progression of DN [69].

miRNAs have been involved in TGF-β1 regulation and pathogenesis of DN, with key miRs such as miR-192, miR-200b and miR-200c being upregulated in the glomeruli of two independent diabetic model mice and in mouse mesangial cells treated with TGF-β1 *in vitro* [70,71]. It has been shown that both miR-192 and miR-200b increased the expression of TGF-β1, Col 1α2 and Col4α1 in mice mesangial cells. In fact, TGF-β1 can be autoupregulated by the activation of a miRNA cascade including miR-192 and key miR-200 family members. The expressions of miR-200b and miR-200c were downregulated by *in vivo* inhibition of miR-192, indicating that miR-200b and miR-200c are downstream genes of miR-192 in mice mesangial cells. *In vitro* inhibition of miR-192 with a miRNA inhibitor and *in vivo* inhibition using locked nucleic acid (LNA)-antimiR-192 decreased the expression of miR-200b/c, Col1α2, Col4α1 and TGF-β1 in mouse mesangial cells, and in mouse kidney cortex.

Moreover, podocyte-specific Dicer knockout mice (key enzyme in miRNA biogenesis) have increased proteinuria, and tubular and glomerular abnormalities [72], while *in vivo* knockdown of miR-29c prevents progression of DN [73].

Krupa *et al.* examined miRNA profiling of renal biopsy samples from patients with DN and found that loss of miR-192 expression associates with decreased estimated glomerular filtration rate (eGFR) and increased fibrosis score [74].

Another approach to examine miRNA changes in DN is to measure their concentration in urine. miRNAs are stable in urine, packaged in extracellular vesicles. Using urine miRNA microarray, Delic *et al.* showed that 16 miRNAs were differentially expressed in DN patients compared with healthy donors and T2DM patients without DN. The most strongly upregulated miRNAs in urinary exosomes from DN patients were miR-320c and miR-6068. The authors focused on miR-320c as it was positively correlated with urinary albumin to creatinine ratio

and negatively correlated with eGFR and it may indirectly be involved in TGF-β signaling via targeting TSP-1 [75]. They propose miR-320c as a candidate marker for kidney disease progression in T2DM.

Another group identified a set of 27 miRNAs which were present at significantly different levels in different stages of DN in T1DM patients. More importantly, they identified a signature of ten miRNAs strongly associated with the subsequent development of microalbuminuria, which may serve as prognostic marker for the development of incipient DN [76].

The lncRNA ASncmtRNA-2 has been shown significantly upregulated in kidney of diabetic mouse and positively correlated with expression of profibrotic factor TGFβ1 [77]. Inhibition of radical oxygen species (ROS) production decreased the expression of ASncmtRNA-2, suggesting that ASncmtRNA-2 acts downstream of ROS and may mediate their effects in DN. Knockdown of ASncmtRNA-2 decreased the expression of TGFβ1, thus targeting ASncmtRNA-2 may prove to be effective for the prevention and treatment of DN.

Diabetic retinopathy

Many epigenetic changes have been shown to regulate key pathways associated with the development of diabetic retinopathy (DR; see Table 1). Thus, histone modifications in *MMP-9* and manganese *SOD2* increase oxidative stress in retina, damage the mitochondria and augment capillary cell apoptosis, leading to DR. Antioxidant treatment or overexpression of *SOD2*, encoding the enzyme responsible for scavenging mitochondrial superoxide, protects mitochondrial homeostasis and prevents DR in animal models. Several histone modifications have been described in human and rat retinal *SOD2*, which may explained the decreased SOD2 expression during diabetes conditions [78,79]. Thus, diabetes induced increased H3K9ac, H4K20me3 (repressive mark) and decreases H3K4me (active mark) at the promoter and enhancer of retinal *SOD2*, with concomitant decrease in its expression. Diabetes increased also the expression of SUV420h2, which is the main enzyme trimethylating H4K20, as silencing *SUV420h2* prevents H4K20me3 at *SOD2*. Decreased H3K4me was related to increased recruitment of LSD-1 at *SOD2*, which demethylates H3K4me1, H3K4me2 and H3K9. Regulation of LSD-1 by siRNA protects glucose-induced demethylation of H3K4 at *SOD2* and prevents the decrease in SOD2 expression. All histone modifications were not reversed by lowering blood glucose levels, suggesting their role in 'metabolic memory' [78,79].

The activation of retinal *MMP-9* is another event that damages mitochondrial function and activates apoptotic machinery leading to DR. Human and rat retinas have decreased H3K9me2 (repressive mark) and increased H3K9ac (active mark) and p65 at the retinal *MMP-9* promoter [80]. It has been proposed that the activated LSD1 in diabetes hypomethylates H3K9 at the *MMP-9* promoter and this liberates lysine (K9) for acetylation. H3K9ac opens the chromatin and increases the NF-κB recruitment, activation of *MMP-9* and mitochondrial damage. LSD-1 siRNA prevented glucose-induced demethylation of H3K9me2 and *MMP-9* activation and mitochondrial injury [80].

In this respect, LSD1 inhibitors could prevent or slow the progression of DR by regulating both *SOD2* and *MMP*-9-mediated mitochondrial damage in diabetic patients. Their promising results in cancer should further support their potential benefit in DR.

More recently, the transcription of *MMP-9* has been shown to be regulated also by a dynamic DNA methylation, in which the maintenance enzyme, Dnmt1 inserts a methyl group, while Tet2 demethylates DNA. Despite the increased binding of Dnmt1 to the *MMP*-9 promoter, concomitant activation of Tet2 keeps *MMP*-9 promoter hypomethylated, with concomitant increased transcription in DR [81]. Silencing of *Tet2* prevents hyperglycemia-induced *MMP-9* hypomethylation and transcription, and maintains mitochondrial homeostasis. These results suggest that regulation of the DNA methylation–demethylation machinery could inhibit the development/progression of DR by preventing *MMP-9*-mediated mitochondrial damage. It has been shown recently that the DNA methylation status of retinal *MMP-9* promoter is under the control of oxidative stress. Amelioration of oxidative stress by chemical/genetic approaches regulates retinal *MMP-9* transcription in diabetes through preventing the decrease in methylation level of its promoter region [82].

TXNIP, an endogenous inhibitor of antioxidant thioredoxin, has been also shown to undergo epigenetic modifications in DR. Hyperglycemia induces in retinal endothelial cells (RECs) the expression of *TXNIP* and inflammatory genes such as *Cox2*, *VEGF-A*, and *ICAM1*. H3K9 modifications were involved in TXNIP-induced inflammation. Thus, overexpression of *TXNIP* in RECs abolishes H3K9me3 (repressive mark) and increases H3K9ac (active mark) at proximal *Cox2* promoter [83].

Another contributing factor in DR is the Nrf2, a transcription factor activated by oxidants to regulate multiple downstream antioxidants defense genes, including the Gclc, a rate-limiting enzyme in glutathione synthesis. Nrf2

is retained in the cytosol by its binding to Keap1, but under oxidative stress, it dissociates from Keap1 and is translocated to the nucleus to regulate transcription of antioxidant genes. In DR, although Nrf2 expression is increased, its DNA binding activity and the binding at *Gclc* are decreased. H3K4 methylation at *Gclc* has a major role in the impaired Nrf2 binding at *Gclc*. Diabetes decreased H3K4me1 and H3K4me3, and increased H3K4me2 in the retina and its endothelial cells. Demethylations of H3K4me1 and H3K4me2 are mediated by LSD1, while KDM5A demethylates H3K4me3. The increase in H3K4me2 was due to increased demethylation of H3K4me3 as H3K4me3 demethylase KDM5A was also increased [84]. Beside its role in the antioxidants defense, Nerf2 regulates the expression of some proinflammatory genes like, *NF-κB*, *Cox2* and *NOS*. Additionally, diabetes increases H3K4me1 (active mark) at the *Keap1* promoter, accompanied by increased HMT Set7/9, impeding the movement of Nrf2 to the nucleus and impairing its transcriptional activity [85].

A recent genome-wide analysis of DNA methylation in blood of T1DM patients has identified 349 differential DNA methylation CpG sites, in 233 genes, in patients with proliferative DR. Moreover, they identified differential DNA methylation of 28 CpG sites, in 17 genes, that predicted the development of DR in a prospective cohort, suggesting that DNA methylation may be used as a marker of DR [86].

In addition to DNA methylation and histone modifications, many miRNAs also play a significant role in the development and progression of DR. Upregulation of proangiogenic VEGF, proinflamatory NF-κB and proapoptotic p53 are well-known early pathological changes of DR. Increased production of extracellular matrix proteins, such as FN is another characteristic feature of DR. SIRT1 downregulation in diabetic retina mediates rapid aging, vascular permeability and FN upregulation [87]. Experimental models of DR have suggested involvement of miRNAs in the regulation of many target genes, including NF-κB-responsive miRNAs (such as *miR-146, miR-155, miR-132 and miR-21*), VEGF-responsive miRNA (such as *miR-17–5p, miR-18a, miR-20a, miR-21, miR-31 and miR-133*) and the p53-responsive miR-34 were upregulated in both the retinas and RECs of the diabetic rats [88]; *Oxr1*-responsive miR-200b was upregulated in diabetic retina [89], the protein activator of protein kinase R, p-53-associated cellular protein PACT/RAX-responsive miR-29b was upregulated in diabetic retina [90]; FN-responsive miRNA-146a was downregulated in diabetic retina [91]; SIRT1-targeting miR-23b-3p and miR-195 were increased in human REC [87,92].

A recent cross-sectional case-control study from the EURODIAB Prospective Complications study of 455 T1DM patients has shown a strong association between miR-126 levels and all diabetes complications including proliferative DR [93].

There are also data demonstrating the implication of various miRNAs in the pathogenesis of diabetic macroangiopathy. Maintaining of rat cardiomyocytes in hyperglycemia *in vivo* and *in vitro* significantly increased the levels of miR-1 and miR-206 in cardiomyocytes, and accelerates apoptosis by depleting the cells of Hsp60, an important component of the cell's defense mechanisms against diabetes [94].

Recently, it has been shown that spinal SIRT1 expression and activity is downregulated in diabetes-induced neuropathic pain rats. Upregulation of SIRT1 by the SIRT1 activator, SRT1720, attenuates neuropathic pain by reducing epigenetically the expressions of *mGluR1/5*, which plays an important role in central sensitization and neuropathic pain in the spinal cord [95].

Metabolic memory

Diabetes major clinical trials have shown a long-lasting beneficial effect of an early intensive glycemic control in diabetic patients, a phenomenon referred to as 'metabolic memory'.

Prior experimental studies have shown that short-term hyperglycemic exposure induces long-term epigenetic changes (reviewed in [2]). More recent large human studies confirm these data. Histone post-translational modifications (PTM) profiling of two selected subsets of DCCT/EDIC participants who experienced different rates of complications showed that monocytes from intensive-treated patients had increased H3K9ac (active chromatin mark) compared with conventional-treated subjects with worse glycemic control, and the acetylation level was correlated with prior HbA_{1c} levels [19]. Of the affected gene promoters, over 50% were related to NF-κB pathway. More recently, the same group performs a DNA methylome profiling, using two sets of DNAs collected, at least 16–17 y apart, from the same subsets of DCCT/EDIC participants [66]. They showed persistency of DNA methylation differences over time. 12 annotated differentially methylated loci were identified, including *TXNIP* hypomethylation, known to be associated with hyperglycemia and related complications. These results show that DNA methylation differences during the DCCT persist for several years during the EDIC study and support, together with previous data on promoter histone PTMs, an epigenetic explanation for metabolic memory [66].

The identification of differentially methylated loci, especially *TXNIP*, in peripheral blood cells, can be used as a biomarker for prior hyperglycemia and metabolic memory.

Overall, these results indicate that even transient exposure to hyperglycemia can lead to epigenetic changes in target cells, resulting in long-lasting changes in gene expression associated with diabetic complications.

Epigenetic targets & therapeutic opportunities

New therapeutic strategies are needed to overcome the increasing prevalence of diabetes and its devastating complications.

A large variety of small molecules, inhibitors and/or activators of epigenetic enzymes have proved to erase the diabetes-induced epigenetic changes (Table 3 in [2]). Many of the dietary phenolic phytochemicals, known for their antioxidant activity, also regulate gene expression by modulating epigenetic changes.

HDACi, HAT inhibitors and HAT activators have demonstrated encouraging outcomes.

HDACi proved to be successful epigenetic therapy against hematological malignancies. Thus, suberoylanilide hydroxamic acid (SAHA)/vorinostat, romidepsin and belinostat are approved by the US FDA for the treatment of T-cell lymphoma, while panobinostat for the treatment of multiple myeloma. The substrate of HDACs is not limited to histones. They may catalyze deacetylation of non-histone proteins like: transcription factor, oncoproteins, signal transducers, among others. HDACi have been associated with toxicities, like thrombocytopenia, neutropenia, anemia, diarrhea, nausea, vomiting, constipation and dehydration. Today many isoform- or class-selective inhibitors are being studied in advanced stages of clinical trials to increase potency and circumvent drug toxicity [96].

Cytoprotective effect

Studies have shown that HDACs inhibition protects pancreatic β cells against apoptosis induced by proinflammatory cytokines.

Initial reports demonstrated that inhibition of HDAC activity with broad-spectrum HDACi trichostatin A (TSA) and SAHA (varinostat) partially prevented cytokine-induced β-cell apoptosis [97]. However, they could not restore glucose-stimulated insulin secretion and may be toxic on their own. *In vivo* administration of another broad-spectrum HDACi, ITF2357, prevented hyperglycemia in streptozocin-treated mice [98]. Susick *et al.* provide evidence for another HDACi, THS-78-5, with a significant potential to prevent IL-1β-mediated effects on isolated β cells [99].

It has been shown that only certain HDAC isoforms are important to cytokine-induced β-cell apoptosis and inhibitors selective for HDAC1, 2 and 3, like MS-275 and CI-994 are more effective in suppressing apoptosis and restorative to β-cell function than broad-spectrum inhibitors like SAHA, trichostatin [100]. Inhibition of HDAC3 appeared to be critical for the protective effects of HDACi, while the inhibition of additional isoforms may be toxic to β-cell.

Insulin sensitivity restoration

The class IIa HDAC are highly enriched in skeletal and cardiac muscle, and are well-known regulators of muscle development and phenotype. More recently, they have been implicated in regulation of insulin signaling and glucose metabolism. Thus, knockdown of HDAC5 in human primary muscle cells or pharmacological inhibition with a broad-spectrum class I and II HDACi scriptaid led to increased glucose uptake by increasing GLUT4 expression, independent of PGC-1α expression [101]. Surprising, but not necessarily contradictory, another group showed that treatment of obese diabetic mice with a class I (MS275) – but not a class II (MC156) – selective HDACi increased oxidative metabolism in adipose tissue and skeletal muscle, supporting energy expenditure and weight loss. PGC-1α mediated the effects of class I HDAC inhibition, as silencing of PGC-1α abolished the effect of these compounds, while genetic knockdown of HDAC3 reproduced the effects of class I HDACi on PGC-1α expression [102].

HDACi have proved beneficial effect on diabetic complications. HDAC2 play important role in TGF-β1-mediated kidney fibrosis. TSA prevented TGF-β1-induced morphologic changes, as well as upregulation of collagen type I and downregulation of E-cadherin in human renal proximal tubular epithelial cells [103]. Similarly, vorinostat decreased albuminuria, mesangial collagen IV deposition and oxidative stress [104], and attenuated glomerular hypertrophy in streptozotocin-induced diabetes [105]. Moreover, DR have been prevented with TSA [106], but also by the HAT inhibitors garcinol [107].

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β-cell differentiation

HDACi have proved beneficial effect in driving the differentiation of stem cells toward insulin-producing cells. In particular, TSA allow differentiation of bone marrow stem cells into insulin-producing cells [108], while sodium butyrate with activin A drive human embryonic stem cells (hESC) to differentiate into insulin-producing islets clusters [109]. Moreover, transdifferentiation of fully differentiated human dermal fibroblasts into islet-like cells has been obtained by using two epigenetic modifying compounds, romidepsin (HDACi) and 5-Azacytidine (DNMT inhibitor) [110]. In the embryonic pancreas, a class II HDACi, MC1568 activates Pax4 expression and increases β- and δ-cell mass [4]. The HAT activators, SPV106, re-established acetylation levels of H3K9/14, and the differentiation and proliferation of cardiac mesenchymal cells isolated from D2DM patients [111].

Inhibition of DNMTs has been shown to be effective against many chronic diseases, and has already been approved in the treatment of cancer. Thus, regulation of *LSD1* by its siRNA ameliorated glucose-induced decrease in H3K4me at *Gclc* and *Sod2* genes, and prevented the decrease in their expression [79,84]. Phytochemicals like, curcumin and sulforaphane have been reported to increase Nrf2 expression by demethylating its promoter and decreasing DNMT1 [112,113].

Several small molecules promote β-cell differentiation and/or proliferation by epigenetic regulation of key transcription factors

For example, the DNMTi, 5-AZA increases Ngn3 expression and endocrine differentiation of human pancreatic ductal cells [114]. Similarly, BRD7552 induced increased expression of PDX1, concomitant with increased H3 acetylation and H3K4me3, and decreased H3K9me3 at the *PDX1* promoter in human pancreatic ductal cells [115]. Another small molecule, WS6 promoted β-cell proliferation *in vitro*, in primary islets of both rodent and human, as well as *in vivo*, in the RIP-DTA mouse model of β-cell ablation [116]. Retinoic acid promoted generation of Ngn3+ endocrine progenitor cells and their further differentiation into β cells in human and mouse embryonic pancreatic explants [117], indolactam V increased PDX1+ pancreatic progenitor cells from hESC [118], sodium cromoglicate induced Ngn3+ cells in hESC and mouse embryonic pancreatic explants [119].

Many growth factors promote β-cell differentiation or regeneration, including nicotinamide, EGF, glucagon-like peptide, gastrin, activin A (a member of TGF-β family), betacellulin, hepatocyte growth factor, keratinocyte growth factor, R-Spondin 1, Noggin and exendin-4. Unfortunately, none of them have been translated for islet generation due to their high cost and low yield of islet clusters. Recently, it has been described a 7-factor culture medium optimal for self-renewal and differentiation of adult pancreatic progenitor cells into the three major pancreatic lineages, including insulin-expressing cells, but EGF and nicotinamide were necessary and sufficient [120].

Several natural compounds inhibit epigenetic enzymes

In diabetes, sulforaphane, an organic isothiocyanate found in cruciferous plants such as broccoli, has been shown to protect β-cell damage by suppressing NF-κB pathways [121]. Another intensively studied natural compound is curcumin. Curcumin reduces IR, hyperglycemia and hyperlipidemia by suppressing NF-κB and activating PPARγ [122]. Resveratrol is another natural compound which has been extensively studied for its potential utility in the management of diabetes. Similarly, resveratrol protect β cells by inhibition of cytokine and attenuation of the oxidative stress [123]. Vitamin D has been recently shown to prevent oxidative stress and increase glucose uptake via SIRT1/AMPK/IRS1/GLUT4 cascade in adipose tissue of diabetic mice [124].

Mediterranean diet and physical exercise have been associated with a lower global DNA methylation level [125].

ssRNA analogs complementary to miRNAs (antagomirs) were developed for therapeutic silencing of different miRNAs in animal models, that were critically involved in diabetes and IR. Trajkovski *et al.* administered antagomirs by tail-vein injection to silence both miR-103 and miR-107 in the livers and adipose of two diabetic mouse models. They found that the efficient silence of both miRNAs significantly alleviate hyperglycemia by promoting insulin signaling in the liver and adipose [51]. In December 2015 has begun the first Phase I clinical study RG-125(AZD4076) with *N*-acetylgalactosamine (GalNAc)-conjugated anti-miR-103/107 oligonucleotide for treatment of non-alcoholic steatohepatitis in patients with T2DM/prediabetes. In a similar approach, Choi *et al.* efficiently silenced the expression of miR-34a in the liver of obese mouse model, with significant improvements in glucose tolerance and insulin sensitivity [126]. Intravenous administration of small LNA against miR-802 to HFD-fed mice significantly suppressed miR-802 in the liver and kidney, and improved liver insulin sensitivity [55]. Kurtz *et al.* [127] administered LNAs by tail-vein injection in C57BL6/J mice to inhibit miR-29. Treatment with

the LNA29 significantly reduced plasma lipids by suppressing lipogenic synthesis in the liver, similar with statin therapy.

Intravitreal injections of miRNA-29b, miRNA-200b and miR-146a have been tested in animal models of DR with encouraging results [91,128].

Conclusion & future perspective

In recent years, there has been significant progress in the fields of epigenetics. Many of the studies summarized here are associative or conducted in animal models. Therefore, causative inferences need to be taken with caution at the present time. However, consistent with the current knowledge and progress, the use of epigenetics as a therapeutic approach is apparently becoming a powerful alternative for gene therapy.

The focus of many researchers is to erase histone modifications and restore optimal miRNA levels to improve β-cell function, insulin sensitivity and chronic complications. Several small molecules have proved their ability to erase epigenetic modifications during experimental studies. Just few have been studied yet in clinical studies. Future tactics will likely employ mutated transcription activator-like effectors (TALE) and CRISPR–Cas proteins that are tethered to HDACs, HATs, DNMTs and other epigenetic-altering molecules, and bind specific DNA sequences. Recently, it has been proved targeted *de novo* DNA methylation of the *Cdkn2a* gene by TALE–DNMT [9].

There is need to investigate novel biomarkers to facilitate early prediction and precise diagnosis of diabetes. For example, environmental prenatal exposure to the maternal hyperglycemia or maternal diet, as well as exposure to the obesogenic diet early in the life induces epigenetic changes that predispose to T2DM later in the life. Identification of these markers may allow early life-style optimization before overt diabetes.

Identification of susceptible people to develop T1DM based on epigenetic markers may allow early intervention, like targeting IL-1β with HDACi to prevent β-cell loss or *Cdkn2a* to increase cell replication. Development of selective HDAC3 or HDAC7 inhibitors for their protecting effect on pancreatic β cells is an appealing strategy.

Executive summary

Background

- Epigenetics studies the heritable changes in gene expression and phenotype that are not caused by changes in the DNA sequence.
- The most studied epigenetic modifications include: DNA methylation, histone modifications and noncoding RNAs.
- Epigenetic regulation of gene expression allows the organism to respond/adapt to environmental changes without changing the DNA sequence itself.
- Epigenetic modifications are responsible for altering gene expression associated with a variety of chronic diseases, including diabetes.

Epigenetic modifications in diabetes

- Epigenetic regulation is critical for β-cell differentiation, cytoprotective effect during inflammatory conditions, tissue-specific insulin expression, glucose-dependent insulin secretion, β-cell insulin content and β-cell mass.
- Epigenetic regulation has important roles in insulin sensitivity.
- Epigenetic repressive modifications at the immune response genes may contribute to the pathogenesis of Type 1 diabetes mellitus.
- Epigenetic marks may allow identification of individuals at increased risk of diabetes/for the development of incipient complications/complications progression.

Therapeutic opportunities

- Histone deacetylase inhibitors counteract diabetes by inducing β-cell differentiation (trichostatin A [TSA], sodium butyrate, romidepsin, MC1568), cytoprotective effect during inflammatory conditions (TSA, suberoylanilide hydroxamic acid, ITF2357, THS-78–5, MS-275, CI-994), restoring insulin sensitivity (scriptaid, MS275) and delaying diabetic complications (TSA, suberoylanilide hydroxamic acid, garcinol).
- The potential therapeutic role of silencing of miR-103, miR-107, miR-143, miR-181, mir-29 and miR-802 to improve glucose homeostasis and insulin sensitivity in Type 2 diabetes mellitus patients with insulin resistance.
- Modulation of miRNA expression levels may be a therapeutic strategy for diabetic complications.
- Erasing metabolic memory of prior hyperglycemia may reduce the risk of chronic complications.
- Many epigenome modifying small molecules have the capability to promote β-cell differentiation and/or proliferation by inducing key transcription factors (like, 5-AZA, BRD7552, WS6, indolactam V, sodium cromoglicate and retinoic acid).
- Many natural compounds have the potential to inhibit the epigenetic enzymes, with beneficial effects in diabetes (like sulforaphane, organic isothiocyanate, curcumin, resveratrol, anacardic acid and vitamin D).

10.2217/pme-2017-0043

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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METABOLIC SYNDROME AND RELATED DISORDERS
Volume XX, Number XX, 2018

Pp. 1–7
DOI: 10.1089/met.2018.0057

Determining Factors of Arterial Stiffness in Subjects with Metabolic Syndrome

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Abstract

Background: Metabolic syndrome (MS) is a clustering entity characterized by obesity, hypertension, hyperglycemia, dyslipidemia, and insulin resistance. Early detection of atherosclerosis is important in patients with MS because cardiovascular diseases are the main cause of mortality in these patients.
Methods: We aimed to investigate the factors influencing arterial stiffness, pulse wave velocity, and the augmentation index, respectively, in 150 subjects with MS (94 women and 56 men; mean age 60.56 ± 9.8 years). Arterial stiffness was measured using the TensioMed™ Arteriograph. We tested the relationship between arterial parameters and insulin resistance measured by the determination of insulinemia (the ELISA method) and the homeostasis model assessment index (HOMA).
Results: In multivariate analysis we identified the independent factors that influence arterial stiffness: systolic blood pressure (coefficient of determination 3.586; $P < 0.0001$), serum triglycerides (coefficient of determination 3.579; $P < 0.0001$), and age (coefficient of determination 3.510; $P = 0.001$) are independent predictive factors for pulse wave velocity. The independent predictive factors of the augmentation index were the body mass index (coefficient of determination 0.55; $P = 0.009$), the presence of diabetes mellitus (coefficient of determination 4.7; $P = 0.03$), mean arterial pressure (coefficient of determination 0.44; $P < 0.0001$), gender (coefficient of determination 9.2; $P < 0.0001$), age (coefficient of determination 0.3; $P < 0.0001$), and heart rate (coefficient of determination 0.66; $P < 0.0001$). Insulin resistance (HOMA index) was a predictor of the brachial augmentation index (β coefficient 3.4; $P < 0.001$) and was not a predictor of pulse wave velocity ($\beta = -0.3$; $P = 0.6$) in our study.
Conclusions: Given the known predictive value of pulse wave velocity for cardiovascular events, identifying the factors responsible for the increase in arterial stiffness is extremely important.

Keywords: arterial stiffness, metabolic syndrome, HOMA index, pulse wave velocity, augmentation index

Introduction

It is important to detect atherosclerosis at an early stage in patients with metabolic syndrome (MS)[1,2] because cardiovascular disorders represent the main cause of mortality in these patients.[3] These pathologies are associated with more cardiovascular risk factors,[4] more comorbidities, and more renal, cardiac,[5] and vascular target organ damage. They are also associated with premature vascular aging, with increased arterial stiffness.[6–10]

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MS is a cluster of inter-related metabolic disorders and vascular risk factors that are closely connected to insulin resistance; it is associated with a twofold increased risk of cardiovascular diseases (CVDs), CVD mortality, and stroke, and a 1.5-fold increased risk of all-cause mortality.[11] There are still debates about the mechanism responsible for enhancing cardiovascular risk in this syndrome, which may involve abnormalities of vascular structure and function. Morphological and functional changes in the arterial wall, such as increased intima–media thickness and vascular wall stiffness, are associated with the presence of cardiovascular risk factors and atherosclerotic disease, being recognized as significant independent predictors of adverse cardiovascular events.[4,5]

Given the known predictive value of pulse wave velocity for cardiovascular events, identifying the factors responsible for the increase of arterial stiffness is extremely important.[12,13]

There is strong evidence that the main determinants of pulse wave velocity are age and blood pressure (BP), and its reference values have been recently established depending on the two parameters.[14] Except for age and BP, which are well-established factors, the other factors that may influence arterial stiffness are extremely controversial in the literature.

Arterial stiffness of the large arteries can be investigated through the development of readily available noninvasive assessment techniques.[15,16] Among different methods, carotid to femoral pulse wave velocity (PWVAo) has emerged as the gold standard because of its relative simplicity and reliability and its association with all-cause and cardiovascular morbidity and mortality.[17–19]

Marked atherosclerosis can represent an important link between the presence of MS and the higher frequency of cardiovascular events.[20] However, atherosclerosis is currently considered an ''inflammatory disease,''[21] inflammation being involved in all stages of atherosclerosis (increase of endothelial permeability—maturation of atherosclerotic plaque—plaque rupture).[21–24]

The connection between inflammation and the arterial system is multifaceted, the endothelium contributing to the initiation and perpetuation of arterial wall inflammation while being affected by the inflammatory cascade.[21]

Aim of the Study

To identify the factors influencing arterial stiffness, pulse wave velocity, and the augmentation index in subjects with MS.

Subjects and Methods

The study was conducted on 150 patients with MS, diagnosed according to international diabetes federation (IDF) criteria.[25] Anthropometric measurements were performed in all patients, and their biochemical and arterial parameters were assessed. Arterial parameters were measured with the TensioMed™ Arteriograph (AG). The AG relies on an oscillometric method using the occlusion technique. Patient data and the distance measured between the jugulum and the symphysis were recorded in an AG-programmed computer (TensioMed Software v.1.9.9.2; TensioMed, Budapest, Hungary). This technique is based on the generation of two systolic peaks. First, an early systolic pressure peak (P1) is created by the ejection of the blood from the left ventricle into the aorta. The pressure wave is transmitted to the lower body and the wave reflected from the periphery (assumed average around the aortic bifurcation) generates the late systolic peak (P2). Pulse pressure (PP) is the difference between the systolic and diastolic BP (in mmHg). Both the aortic augmentation index (AixAo) and the brachial augmentation index (Aixb) are calculated as $100 \times (P2 - P1)/PP$. The return time (RT) is the time difference (in ms) between the first systolic wave (P1) and the reflected systolic wave (P2) and is related to aortic stiffness. PWVAo was calculated as the distance between the jugulum and the symphysis (meters), divided by the return time (RT/2) (seconds). PWVAo and AixAo are expressed as mean values from two recordings. AG does not automatically show Aix normalized to heart rate 75 (Aix@HR75). For PWVAo, two recordings with the lowest standard deviations were selected. AG was validated and compared with invasive techniques.[26]

According to IDF criteria for the diagnosis of MS, the presence of abdominal obesity is mandatory (abdominal circumference ≥94 cm in men and ≥80 cm in women), in conjunction with two of the following abnormalities: BP >130/85 mmHg, serum triglycerides (TG) ≥150 mg/dL, high-density lipoprotein (HDL) cholesterol <40 mg/dL in men and <50 mg/dL in women, and serum glucose ≥100 mg/dL.

Anthropometric measurements, including weight, height, and waist circumference, were carried out. The body mass index (BMI) was calculated based on anthropometric measurements. Hypertension was diagnosed using BP measurements taken at least twice during two or three different appointments, in a quiet room, after a 15-min rest in a lying position. Type 2 diabetes or impaired fasting glucose was diagnosed based on WHO criteria. TG, total cholesterol, low-density lipoproteins (LDLs), and HDLs were assessed according to standard protocols.

All patients signed an informed consent to participate in the study, and the approval of the ethics committee was obtained.

The factors that correlated with arterial parameters in subjects with MS were identified. Pearson's correlation test was used for quantitative variables with a normal distribution, and Spearman's correlation test was used for quantitative variables with an abnormal distribution.

We tested the relationship between arterial parameters and insulin resistance measured by the determination of insulinemia (the ELISA method) and the homeostasis model assessment index [HOMA = insulin (μU/mL)*glycemia (mg/dL)/405].

Linear regression was used to study the main determining factors of arterial stiffness.

In multivariate analysis (the stepwise method), independent factors that influence arterial stiffness were identified. A multiple linear regression model was developed for PWVAo and for AixAo after a selection of variables starting from the initial model, using the stepwise technique. Stepwise multivariate linear regression was conducted to investigate the predictive effect of age and baseline risk factors for arterial stiffness. Risk factors associated with a P value <0.05 in the univariate analysis were included in the multivariate regression model.

The dependent variable was PWVAo, whereas the covariates were systolic BP, age, and serum TG. For AixAo, the covariates were gender, BMI, presence of diabetes mellitus, mean arterial pressure, age, and heart rate.

Results

The patients included in the study had a mean age of 60.56 ± 9.8 years, and their gender distribution was 94

Table 1. Baseline Characteristics of the Study Group

Parameter	*Female,* N = *94*	*Male,* N = *56*	P
Age	60.15 ± 9.6	61.2 ± 10.18	NS
BMI, kg/m^2	29.28 ± 5.26	30.58 ± 3.87	0.08
Weight, kg	79.43 ± 15.08	95.36 ± 12.95	<0.0001
Waist circumference, cm	98.69 ± 13.33	108.75 ± 9.47	<0.0001
Glycemia, mg/dL	104.04 ± 26.25	113.31 ± 29.12	0.05
LDL, mg/dL	140.66 ± 38.55	120.05 ± 35.74	0.001
HDL, mg/dL	43.51 ± 8.26	38.71 ± 9.35	0.001
TG, mg/dL	169.16 ± 81.26	192.51 ± 86.51	NS
SBP, mmHg	138.76 ± 31.62	133.64 ± 38.69	NS
DBP, mmHg	83.95 ± 16.65	80.96 ± 23.62	NS
PWVAo, m/s	9.87 ± 2.05	10.12 ± 1.93	NS
AixAo	40.13 ± 14.38	33.16 ± 13.94	0.004
Aixb	6.05 ± 26.66	–6.16 ± 27.93	0.009
PP, mmHg	54.84 ± 17.68	51.05 ± 17.56	NS

Results are expressed as mean values ± standard deviations.

AixAo, the aortic augmentation index; Aixb, brachial augmentation index; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PP, pulse pressure; PWVAo, pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides.

women and 56 men. The baseline characteristics of the study group are shown in Table 1.

The correlation between the components of MS (abdominal obesity, arterial hypertension (AHT), dyslipidemia, and hyperglycemia) and arterial parameters (carotid–femoral pulse wave velocity, the Aixb, and the AixAo) was studied. Among MS components, weight, BMI, and abdominal circumference were statistically significantly correlated (Table 2) with the AixAo and the Aixb and were not correlated with carotid–femoral pulse wave velocity.

As shown in Table 2, all arterial function parameters, AixAo, AixAb, and PWVAo, were correlated positively with systolic, diastolic BP, mean arterial pressure, and PP.

Regarding dyslipidemia and glycemic values, their presence was found to be correlated differently with each arterial function parameter. Thus, the Aixb was significantly correlated with the value of HDL cholesterol and was not correlated with the values of total cholesterol, LDL cholesterol, TG, and glycemia, and the AixAo was significantly correlated with the values of total cholesterol and was not correlated with the values of TG, LDL cholesterol, and glycemia. Carotid–femoral pulse wave velocity was significantly correlated with the value of TG and was not correlated with the values of glycemia, total cholesterol, LDL cholesterol, and HDL cholesterol.

Linear regression was used to analyze the main determining factors of arterial stiffness.

In this study, a linear relationship between pulse wave velocity and the age of patients with an R^2 of 0.063 ($P = 0.001$) was observed, age being an independent predictor of arterial stiffness in subjects with MS. The other predictors of arterial stiffness were studied by univariate regression. Thus, other independent predictors of pulse wave velocity were systolic BP ($\beta = 0.344$; $R^2 = 0.118$; $P < 0.0001$), diastolic BP ($\beta = 0.148$; $R^2 = 0.022$; $P = 0.032$), mean arterial pressure ($\beta = 0.200$; $R^2 = 0.04$; $P = 0.003$), PP ($\beta = 0.198$; $R^2 = 0.038$; $P = 0.004$), and TG ($\beta = 0.206$; $R^2 = 0.04$; $P = 0.003$).

In univariate regression, BMI, abdominal circumference, glycemia, total cholesterol, LDL cholesterol, and HDL cholesterol were not independent predictors of pulse wave velocity.

Of all considered parameters (age, BMI, abdominal circumference, systolic BP, diastolic BP, PP, glycemia, total cholesterol, HDL cholesterol, LDL cholesterol, and TG), systolic BP was the most important determinant of pulse wave velocity ($R^2 = 0.756$; $P < 0.0001$).

In multivariate analysis (the stepwise method), we identified the independent factors that influence arterial stiffness.

Table 3 presents the independent predictive factors of pulse wave velocity (model summary and coefficients). It can be seen that systolic BP (coefficient of determination 3.586; $P < 0.0001$), serum TG (coefficient of determination 3.579; $P < 0.0001$), and age (coefficient of determination 3.510; *P*-0.001) are independent predictive factors for pulse wave velocity.

A multiple linear regression model was developed for AixAo after a selection of variables starting from the initial model, using the stepwise technique (Table 4). The independent predictive factors of the AixAo were BMI, the presence of diabetes mellitus, mean arterial pressure, gender, age, and heart rate.

We tested the relationship between arterial parameters and insulin resistance measured by determination of insulinemia and the HOMA index. The Aixb correlated negatively with insulin resistance (HOMA –0.19; $P = 0.04$) and the AixAo did not correlate with insulin resistance (HOMA –0.16; $P = 0.09$). PVWAo did not correlate with insulin resistance (HOMA –0.04; $P = 0.6$; insulinemia 0.07; $P = 0.4$).

Table 2. Correlation Between Arterial Function Parameters and Biological, Hemodynamic, and Anthropometric Variables

	Age	*Weight*	*BMI*	*SBP*	*DBP*	*MAP*	*PP*	*Glycemia*	*Cholesterol*	*LDL*	*HDL*	*TG*
Aixb												
Correlation coefficient	0.358	–0.453	–0.332	0.334	0.195	0.448	0.409	–0.065	0.129	0.093	0.305	–0.045
P	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	<0.001	0.352	0.064	0.182	<0.001	0.516
AixAo												
Correlation coefficient	0.345	–0.432	–0.318	0.353	0.221	0.456	0.401	–0.056	0.150	0.112	0.305	–0.030
P	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	0.425	0.031	0.107	<0.001	0.665
PWVAo												
Correlation coefficient	0.252	0.035	0.097	0.344	0.148	0.270	0.241	0.089	0.099	0.025	0.008	0.206
P	<0.001	0.611	0.163	<0.001	0.032	<0.001	0.001	0.203	0.154	0.723	0.910	0.003

Aixb, brachial augmentation index; AixAo, aortic augmentation index; BMI, body mass index; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; PWVAo, pulse wave velocity; SBP, systolic blood pressure.

TABLE 3. MULTIVARIATE ANALYSIS FOR PWVAO: MODEL SUMMARY AND COEFFICIENTS

Model summary[a]

Model	R	R^2	*Adjusted* R^2	*Standard. error of the estimate*	*Change statistics*					*Durbin–Watson*
					R^2 *change*	*F change*	*df1*	*df2*	*Sig. F change*	
1	0.370[b]	0.137	0.131	1.3410	0.137	25.217	1	159	0.000	
2	0.432[c]	0.187	0.177	1.3056	0.050	9.731	1	158	0.002	
3	0.496[d]	0.246	0.232	1.2612	0.059	12.322	1	157	0.001	1.975

Coefficients

Model		*Unstandardized coefficients*		*Standardized coefficients*			*95% CI for* B		*Correlations*			*Collinearity statistics*	
		B	*Std. error*	Lβ	t	*Sig.*	*Lower bound*	*Upper bound*	*Zero order*	*Partial*	*Part*	*Tolerance*	*VIF*
1	Constant	5.755	0.856		6.726	<0.0001	4.065	7.444					
	SBP, mmHg	0.031	0.006	0.370	5.022	<0.0001	0.019	0.043	0.370	0.370	0.370	1.000	1.000
2	Constant	5.018	0.866		5.795	<0.0001	3.308	6.728					
	SBP, mmHg	0.031	0.006	0.369	5.147	<0.0001	0.019	0.043	0.370	0.379	0.369	1.000	1.000
	TG, mg/dL	0.005	0.002	0.224	3.120	0.002	0.002	0.008	0.225	0.241	0.224	1.000	1.000
3	Constant	3.613	0.927		3.896	<0.0001	1.781	5.444					
	SBP, mmHg	0.023	0.006	0.269	3.586	<0.0001	0.010	0.035	0.370	0.275	0.248	0.855	1.170
	TG, mg/dL	0.005	0.002	0.249	3.579	<0.0001	0.002	0.008	0.225	0.275	0.248	0.989	1.011
	Age, years	0.042	0.012	0.264	3.510	0.001	0.018	0.065	0.343	0.270	0.243	0.847	1.181

[a]Dependent variable: PWVAo.
[b]Predictors: constant, SBP.
[c]Predictors: constant, SBP, TG.
[d]Predictors: constant, SBP, TG, age, years.
CI, confidence interval; PWVAo, pulse wave velocity; SBP, systolic blood pressure; TG, triglycerides; VIF, variance inflation factor (multicollinearity test).

In univariate regression, we investigated whether insulin resistance was a predictor of arterial stiffness. We introduced into the regression model only variables correlated with one another.

Insulin resistance (HOMA index) was a predictor for the Aixb ($\beta = 3.4$; $P < 0.001$) and was not a predictor for pulse wave velocity ($\beta = -0.03$; $P = 0.6$) or for the AixAo ($\beta = -1.1$; $P = 0.09$) in our study.

Discussion

MS induces an increase in arterial stiffness independently of other cardiovascular risk factors.[27]

Most studies have shown that pulse wave velocity is independent compared with the majority of classical risk factors for atherosclerosis and is not associated with gender, smoking, and dyslipidemia.[12]

TABLE 4. TABLE OF COEFFICIENTS OF THE MODEL CHOSEN BY THE STEPWISE METHOD FOR AORTIC AUGMENTATION INDEX

	B	*95% CI*	P
Intercept	33.5846497257396	11.2737–55.8956	0.003
BMI	–0.5581	–0.9756 to –0.1406	0.009
DM_NO	4.7064	0.5402–8.8727	0.03
MAP	0.4437	0.3283–0.5591	<0.001
SEXM_NO	–9.2788	–12.8533 to –5.7043	<0.001
Age	0.3063	0.1325–0.4801	<0.001
HR	–0.6671	–0.8304 to –0.5037	<0.001
LDL	0.0389	–0.0083 to 0.0861	0.11

BMI, body mass index; DM, diabetes mellitus; HR, heart rate; LDL, low-density lipoprotein; MAP, mean arterial pressure.

It is known that age is an important factor that influences arterial stiffness in the general population; arteries become increasingly stiff with aging.[28] There are insufficient data about the factors that might influence the value of pulse wave velocity in patients with MS.

In this study, a linear relationship between pulse wave velocity and the age of patients can be observed. Furthermore, age is an independent predictor of arterial stiffness in subjects with MS in the multivariate regression model. The other determining factors of arterial stiffness are systolic BP, diastolic BP, mean arterial pressure, and PP, which are reported in the literature as predictors of pulse wave velocity.

Important evidence has shown that sustained arterial hypertension accelerates atherosclerosis[29] and induces arterial smooth muscle hyperplasia and hypertrophy, which leads to an increase in arterial stiffness.[30] It is possible that pulse wave velocity may correlate more closely with the duration and severity of arterial hypertension than with the BP value measured during the evaluation of pulse wave velocity. Thus, pulse wave velocity can be a better ''measure'' of BP than its conventional measurement.[12]

The association between glycemia and pulse wave velocity is controversial in the literature, the majority of the studies demonstrating that risk factors for atherosclerosis, excepting arterial hypertension, are not associated with an increase in pulse wave velocity.[31,32]

In a study Henry et al. found the following determining factors of arterial stiffness: abdominal circumference, BP, and glycemia.[33]

In this study, no correlation was found between glycemia and arterial stiffness parameters, such as pulse wave velocity, the AixAo, and Aixb, which is in accordance with the data presented by other authors.[34] The relationship between glycemia and arterial stiffness still has many unknown aspects and requires further studies.[35]

Regarding the connection between the level of cholesterol and pulse wave velocity, interestingly, statin trials had a positive effect,[36] but also a negative effect on pulse wave velocity.[37] This study also found no correlation between the cholesterol level and pulse wave velocity. The only lipid parameter that was correlated with pulse wave velocity in this study was the level of TG. In contrast, a good correlation between HDL cholesterol and the AixAo and Aixb was observed.

Chen et al.[38] showed in a study on an Asian population that arterial stiffness was correlated with all MS components except for HDL cholesterol in men. The strongest correlation was observed between arterial stiffness and BP, basal glycemia, respectively. The same authors described a correlation between arterial stiffness and advanced age and the number of MS components.[38,39]

Obesity also has adverse effects on the vascular system by increasing arterial stiffness.[40,41] Factors that have been identified as risk factors for arterial stiffness include visceral fat and abdominal circumference.[42] The increasing prevalence of obesity, which induces an increase in arterial stiffness, has an overwhelming importance in public health strategies.

Unlike these two studies and in accordance with the majority of published studies, we found no correlation between obesity and pulse wave velocity.

In contrast, all parameters that define obesity were negatively correlated with the AixAo and Aixb (weight, BMI, and abdominal circumference). Janner et al.[43] reported that the determining factors of the augmentation index are age, heart rate, and weight and proposed two different equations, depending on gender, for the real calculation of AIx [men: $AIx = 79.20 + 0.63$ (age) -0.002 (age^2) $-$ 0.28 (heart rate) -0.39 (weight); women: $AIx = 56.28 + 0.90$ (age) -0.005 (age^2) $-$ 0.34 (heart rate) -0.24 (weight)]. In this study, the independent predictive factors of the augmentation index were the BMI, the presence of diabetes mellitus, mean arterial pressure, gender, age, and heart rate.

Sipilä et al.[27] showed that systolic BP, age, abdominal circumference, and the basal glycemia level are independently associated with the increase of pulse wave velocity. In this study, only systolic BP, age, and serum TG were associated with an increase in pulse wave velocity, independently of other factors. In a multivariate regression model, we established that systolic BP, TG, and age were independent predictive factors of pulse wave velocity. These data are similar to the results obtained by other authors, particularly for age and BP. The independent predictive value of TG for pulse wave velocity was demonstrated in a few studies.[44,45]

The same authors maintain that the association between MS and arterial stiffness is mediated by insulin resistance. In this study, we found a negative correlation between insulinemia, HOMA, and the Aixb; a less significant correlation between the AixAo and insulin resistance; and no correlation between pulse wave velocity and insulin resistance. In univariate regression, the HOMA insulin resistance index was a predictor of the Aixb and was not a predictor of pulse wave velocity, as shown by other authors. Henry indicated that an increase in arterial stiffness in subjects with MS is independent of the HOMA insulin resistance index.[33]

Scuteri et al.[46] demonstrated that arterial stiffness and consequently pulse wave velocity evolve in parallel to the decrease in insulin sensitivity, before hyperglycemia or arterial hypertension becomes obvious, and that arterial stiffness in the first-degree relatives of normoglycemic normotensive subjects with type 2 diabetes mellitus is more obvious in the presence of insulin resistance.

Limitations of the study

Our study has some limitations: the small number of patients and the evaluation of arterial stiffness by a method that is not the gold standard.

Conclusions

Systolic BP, TG, and age are independent predictive factors for pulse wave velocity in subjects with MS. In our study, insulin resistance was a predictor of the Aixb and was not a predictor of pulse wave velocity.

The independent predictive factors of the AixAo were BMI, the presence of diabetes mellitus, mean arterial pressure, gender, age, and heart rate.

Practical Implications

When the predictive factors of arterial stiffness in MS are known, we can reduce cardiovascular events through aggressive control of these factors.

Authors' Contributions

All the authors contributed to the conception and design of the article. All of the authors approved the final version submitted for publication.

Author Disclosure Statement

No conflicting financial interests exist.

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in vivo *33*: 1051-1058 (2019)
doi:10.21873/invivo.11573

Review

Pharmacogenetic Implications of eNOS Polymorphisms (*Glu298Asp*, *T786C*, *4b/4a*) in Cardiovascular Drug Therapy

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Abstract. *Endothelial nitric oxide synthase (NOS3 or eNOS) is the enzyme responsible for the highest production of nitric oxide, with the greatest impact on the cardiovascular system, encoded by the eNOS gene, which presents various polymorphisms. ENOS gene polymorphisms play an important role in the response to drugs affecting nitric oxide (NO) signaling. This review discusses the pharmacogenetic impact of eNOS polymorphisms on the response to drugs affecting NO activity: angiotensin converting enzyme inhibitors, angiotensin II receptor antagonists, calcium blockers, beta-blockers, diuretics, phosphodiesterase inhibitors, and statins. The identification of biomarkers that accurately predict particular phenotypes is a challenge that needs additional large studies, in different populations. Efforts should be oriented towards a more accurate evaluation of the effects of eNOS genetic variants on biochemical parameters reflecting eNOS gene expression and enzymatic activity, in different diseases, as well as following drug treatment. This approach will allow for a better understanding of the role of eNOS genetic variants in cardiovascular disease progression and for cardiovascular drug therapy optimization.*

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Key Words: Cardiovascular drug, eNOS, pharmacogenetics, polymorphisms, nitric oxide, review.

Lack of a positive response to drugs or a toxic reaction to usual doses are responsible for high morbidity and mortality rates and for increasing health care costs (1, 2). The variability of patients' response can be explained by pharmacogenetics, an interdisciplinary field combining pharmacological and genetic information, which addresses how genetic polymorphisms may influence the response or lack of response to different classes of drugs, as well as the development of various degrees of toxicity (1, 3). Previous studies have showed that 20-95% of the variation in individual drug responses can be explained by genetic polymorphisms (4).

In the cardiovascular field, the response to administrated medications is influenced by many factors, such as doses, patient's disease and particularities (dyslipidemia, hypertension, atherosclerotic diseases, genetic polymorphism) (5-7).

Endothelial dysfunction represents one of the first steps of atherosclerosis, which develops even before any angiographic evidence of disease. One basic characteristic of this dysfunction involves the alteration of nitric oxide (NO) synthesis, release and activity (8).

The production of NO is mediated by the endothelial nitric oxide synthase (eNOS) (9, 10) from L-arginine by NOS (neuronal, endothelial and inducible) (10). Decreased NO synthesis results in the development of cardiovascular diseases, responsible for the increased number of deaths worldwide (11). Various eNOS gene polymorphisms have been described (10), such as insertions/deletions, microsatellites, single nucleotide polymorphisms (SNPs), and variable number of tandem repeats (VNTRs).

NO signaling can be modulated by different drugs, and their effect may be influenced by eNOS gene polymorphism (12-18). Despite being constitutively expressed, eNOS is regulated by many stimuli at transcriptional, posttranscriptional and posttranslational level (19). In this regard, variations in the *eNOS* gene influence its activity, which consequently affects NO production (20).

NO plays an important role in the normal physiology of the cardiovascular system, and dysfunctional NO signaling has been associated with development and progression of cardiovascular diseases – myocardial infarction, coronary spasm, intra-stent thrombosis, heart failure (21-25, 26-31).

The following eNOS gene polymorphisms, as the most extensively studied, will be described here: i) *G894T* (*Glu 298Asp*), exon 7 (rs1799983), ii) *T786C*, promoter SNP (rs2070744), and iii) Intron 4 (*4b/4a*), VNTR.

The single nucleotide polymorphism, rs1799983 is located in exon 7, position 894 in the NOS3 gene (G894T) and determines a glutamine to aspartate change at position 298 of the protein (*Glu298Asp*) (32). The variant allele for *G894T* polymorphism reduces eNOS binding to caveolin-1, leading to eNOS diminished availability and activity in endothelial cells (33). The presence of reduced NO formation in patients with this allelic variant supports these *in vitro* findings (34, 35). At a transcriptional level, eNOS can be affected by a single nucleotide polymorphism, rs2070744 (*T786C*), which dramatically reduces ENOS transcriptional activity (36, 37). This effect is linked to replication protein A1 (RPA1), which binds to the eNOS promoter with higher affinity in the presence of the mutation (38). In fact, inhibition of RPA1 expression can restore the transcriptional activity in the eNOS promoter with the C allele, whereas RPA1 over-expression has the contrary effect (38). Interestingly, these *in vitro* studies are consistent with *in vivo* changes, which have shown that the circulating levels of NO-related markers in subjects carrying the C allele tend to be lower (16) compared to that of T allele carriers, supporting a functional importance of this polymorphism (38).

The *4b/4a VNTR* polymorphism in intron 4 (variable number of tandem repeats on intron 4) of the eNOS gene regulates eNOS post transcriptionally, by altering the formation of a small interfering RNA (siRNA) (39). The types of this polymorphism with four (variant 4a) or five copies (variant 4b) of the 27 bp siRNA fragment (20) are the most commonly met alleles. *In vitro* studies have shown higher siRNA levels in endothelial cells containing five copies, determining lower eNOS mRNA levels, compared to cells containing only four copies (39, 40).

Correlations between eNOS gene polymorphisms and differentiated responses to cardiovascular drugs have also been reported (41). In this review we discuss the pharmacogenetic impact of eNOS polymorphisms on drugs that affect eNOS activity, such as antihypertensive drugs with a role in NO bioavailability. These include: i) angiotensin converting enzyme inhibitors, ii) angiotensin II receptor antagonists, iii) calcium blockers, iv) beta-blockers, v) diuretics, and other drugs whose action is influenced by nitric oxide, such as phosphodiesterase inhibitors and statins.

Angiotensin-converting Enzyme Inhibitors

Antihypertensive drugs' effects are influenced by ENOS polymorphism (14, 18). Angiotensin-converting enzyme (ACE) inhibitors are among the most used antihypertensive drugs (42). ACE inhibition is responsible for vasodilation and improvement of endothelial function, through decreasing the levels of angiotensin II and increasing those of bradykinin (43). Bradykinin stimulates endothelial cell receptors, leading to eNOS activation, NO release and vasodilation (44).

Silva *et al*., showed in a study on hypertensive patients treated with enalapril (an ACE inhibitor), that the "C" alleles of the rs2070744 polymorphism were more frequently found in patients with a good response to Enalapril (18). On the other hand, the same group has also shown that there were no differences between responders and non-responders to enalapril when they beared the *4b/4a* and *Glu298Asp* alleles of the eNOS polymorphism (18).

Sandrim *et al*., reported that the "C Glu b" haplotype is more frequently found in NT (normotensive healthy controls) compared to HT (hypertensive patients) or RHT (resistant hypertensive patients) (NT 21% *versus* HT 8% and RHT 7%, both $p<0.00625$) (45). In HT or the RHT groups, the "C Asp b" haplotype was more frequently found (HT 22% and RHT 20%, *versus* NT 8%, both $p<0.00625$) (45). According to this study, no significant difference was found between HT and RHT patients regarding distribution of eNOS haplotypes in the HT and RHT groups, suggesting an inconsequential influence of the eNOS gene on the antihypertensive therapy's resistance.

Oliveira-Paula *et al*., (46) also reported that GG genotype for rs16960228 polymorphism of the PRKCA influenced the quality of enalapril response. In a recent review, Luizon *et al*. (47), showed that eNOS polymorphisms are associated with susceptibility of preeclampsia (PE) and affect the response to antihypertensive treatment in PE. In fact, eNOS haplotypes combining the "C, a, Glu" of the T-786C (rs2070744), 27 bp VNTR a/b and Glu298Asp (rs1799983) were more frequent in the responsive subgroup of PE to antihypertensive treatment (47).

Angiotensin II Receptor Blockers (ARBs)

Clinical studies have confirmed a significant amelioration of the endothelium-dependent vasodilation in patients with arterial hypertension treated with angiotensin II receptor

blockers (ARBs) compared to placebo or other antihypertensive agents (48-51). The increased NO release from the ARB therapy can be pharmacologically explained by the reduced activity of angiotensin II, resulting in enhanced antioxidant protection and NO bioavailability (52, 53). Even though bradykinin concentration is not increased by ARBs,the latter can induce eNOS expression (54-56). Mason *et al*., (55) have reported higher endothelial NO release induced by all the tested ARBs (losartan, olmesartan, telmisartan, valsartan) compared to untreated cells. Different ARBs affect the NO release in various ways, depending on eNOS polymorphisms. Olmesartan (55) can raise NO levels by approximately 30 % in different eNOS genotypic backgrounds, with the most evident difference in ECs from donors homozygous for the nucleotide mutations *T-786C* and *G894T*. This result shows the specific effects of ARBs on eNOS function, which is in turn influenced by single nucleotide substitution, followed by changes in NO metabolism and finally leading to augmented cardiovascular risk, including hypertension (57-62). The relative contribution of the different mutations to ARB responses requires a separate thorough study.

Beta-blockers

Liljedahl *et al*., have demonstrated that the G allele of the *2996A/G eNOS* polymorphism is associated with a higher blood pressure (BP) response to a β-blocker, and the A allele of the *498G/A eNOS* polymorphism is associated with higher BP responses to a β-blocker and an angiotensin II receptor blocker (63).

Pacanowski *et al*., have shown that patients with eNOS polymorphisms have relatively lower BP during treatment and enhanced rates of BP control (64). Although this correlation was important only for patients assigned to the verapamil SR strategy, a similar tendency appeared when alternative treatment using atenolol was administered. Thus, aggressive multi-drug regimens used in INVEST (International Verapamil SR/Trandolapril Study) (65), irrespective of the drug types included, may induce a superior therapeutic response in patients with the *-786T>C* polymorphism. INVEST was a prospective, randomized trial comparing antihypertensive therapy with β-blockers *versus* calcium antagonists in 22,576 CAD (coronary artery disease) patients with hypertension (65). The accurate mechanism of the stronger association observed in subjects treated with verapamil is not known.

Interestingly, in agreement with prior evidence, this result supports the fact that atenolol does not influence endothelial function or alteration of NO biodisponibility (66-67). A difference of 4-7 mmHg in BP across the different genotype groups in the verapamil SR arm is a clinically significant variation, knowing the fact that a constant reduction of 12 mmHg in systolic BP prevents one death for every 11 patients treated (68). There is no consistent association of eNOS polymorphisms with BP phenotypes (69-71). These results suggest that eNOS genotype might contribute to heterogeneity of responses to several antihypertensive drugs, and pharmacogenetic studies should pursue a more complete characterization of this biologically plausible polymorphism drug relationship.

Calcium Blockers

Several calcium blockers, such as nifedipine, have shown to improve both endothelial function and NO bioavailability (72-73). According to previous studies, amlodipine (a calcium blocker) may provoke vasodilation through eNOS activation (74). At least one of the pathways in eNOS activation is known to be calcium-dependent (72-73). Amlodipine blocks Ca efflux, deactivating eNOS through regulation of Ca. In a randomized clinical trial, Zhang *et al*., reported that for rs1799983 (*Glu298Asp G>T*), lower all-cause mortality was observed in minor allele carriers treated with amlodipine *versus* lisinopril [for GG HR=1.01 (95%CI=0.91-1.13), GT+TT=0.85 (95%CI=0.75-0.97), p=0.04] (75). The authors also showed significant associations between eNOS variants with CHD and heart failure as well as significant pharmacogenetic effects for stroke and all-cause mortality. This suggests the fact that eNOS3 polymorphisms might give practical information with regard to treatment choosing decisions in the future.

Diuretics

Thiazide diuretics are extensively used for hypertension control, with hydrochlorothiazide (HCTZ) being the most used in the clinical practice (76). It has been established that the *Glu298Asp* polymorphism in the *eNOS* gene is capable to modulate the response to hydrochlorothiazide, thus subjects homozygous for the *Glu* allele have a significantly greater reduction in BP levels compared to individuals with the *Asp* allele (76). Despite having a significant effect, it is rather is small, demonstrating that, for a single polymorphism, it is difficult to explain the entire genetic background underlying variations in the pharmacological responses of a medication class.

Phosphodiesterase Type 5 (PDE-5) Inhibitors

eNOS polymorphisms also influence the treatment of erectile dysfunction (ED) (15, 77). Phosphodiesterase type 5 (PDE-5) inhibitors are commonly used for the treatment of ED since inhibition of the PDE-5 enzyme increases the tissue cGMP concentration, mainly in the absence of NO signaling (77). Eisenhardt *et al*., have shown that homozygous subjects

for the "Asp" allele of the *Glu298Asp* eNOS polymorphism are less responsive to sildenafil (an PDE-5 inhibitor) (13), however, this association is still subject to controversy (13, 15). Patients with ED carrying the "4a" and "C" alleles show enhanced responses to sildenafil *versus* the "4b"and "T" alleles for the *4b/4a* VNTR and *g.-786T>C* eNOS polymorphisms, respectively (13, 15). Muniz *et al.*, have reported that reduced plasma nitrite concentration predicts better responses to sildenafil. In addition, response to sildenafil is influenced by eNOS haplotypes including *g.-786T>C*, *Glu298Asp* and *4b/4a* VNTR (15).

Lacchini *et al.*, have demonstrated that the "T" allele of the *Glu298Asp* eNOS polymorphism, the "4b" allele of the *4b/4a* VNTR and the "C" allele (*T786>C*) are associated with a poor response to therapy with PDE-5 inhibitors (77).

In a recent study by Di Salvatore *et al.*, the role of eNOS polymorphism in the development of hypertension and proteinuria was presented, by associating the eNOS *c.-894T* genotype with a significantly higher rate of grade 3-4 hypertension and proteinuria (p=0.0002) (78).

In this context, carriers of the "T" allele of *c.-894T* eNOS polymorphism that show lower basal eNOS levels may have increased risk of hypertension induced by VEGF blockade (79-81).

Statins

The main effects of statins are due to the decrease in cholesterol synthesis (8), through blocking of the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate (8, 10, 82-83), independent of eNOS polymorphisms.

At the same time, as it has previously been suggested (10, 41) that statins' effects and eNOS polymorphisms modulate each other in a reciprocal way. The decrease in serum cholesterol can also cause an increase in endothelial nitric oxide production (8, 82, 84) and improve endothelial function (8), even before a significant decrease in serum cholesterol occurs (85-89).

Statins can affect eNOS regulation at the transcriptional and posttranscriptional level (90). Studies have shown that fluvastatin and atorvastatin can increase the transcriptional activity of the eNOS gene. Statins can also inhibit the replication protein A1 (RPA1), a repressor of the transcriptional activity of eNOS (1, 16, 41), particularly in subjects with the CC genotype (1, 12, 38, 91). This has been demonstrated by increased circulating levels of nitric oxide biomarkers (nitrite and nitrate) following administration of statins, regardless of the genotype, but being higher in C-allele carriers (1, 12, 16, 41, 82, 83). Such effects have been reported in hypertensive (92), dyslipidaemic subjects (93) and obese women (83) so far.

Statins can also increase eNOS expression and activity – compensating the genetic disadvantage of subjects with the CC genotype for the *T786C* polymorphism - (1, 10, 16, 41, 94-98), effect on eNOS expression not being lowered when LDL cholesterol values normalized (94).

Statins play an additional role in eNOS function by decreasing the level and inhibitory activity of caveolin (99, 100). In patients with the eNOS *Glu298Asp* mutation (guanine to thymine conversion at position 894 of the gene), the mutant endothelial cells seem to produce less NO, reducing the availability of eNOS in the caveolae of these cells (41).

Statins inhibit isoprenoids, small GTP-binding proteins, Rho (which induces an increase in vascular smooth muscle sensitivity to calcium), Ras, and Rac (its activation leads to the formation of lamellipodia and membrane ruffles) (8, 89). Therefore, statins cause an accumulation of inactive Ras and Rho in the cytoplasm (8, 89) and increase eNOS expression (101) and eNOS mRNA stability (8, 55, 94), thus prolonging the eNOS mRNA half-life (101).

Statins also bear antioxidant properties, particularly in the CC genotype (1, 8, 16, 83). They decrease different markers of atherosclerosis (8, 82), the anti-inflammatory effect being modulated by eNOS polymorphism (1, 16, 41, 82-83). This idea was demonstrated by a study using atorvastatin, which significantly reduced sCD40L, sVCAM-1, sP-selectin and MMP-9 concentrations only in subjects with the CC genotype (41, 82).

Statins are capable of reducing the fluidity of the red blood cell plasma membrane, but only in the case of the CC genotype for the T786C polymorphism (41, 82).

Stains' modulation on the eNOS system is influenced by the polymorphism in *intron 4*. Adenosine-induced coronary vasodilation was assessed by Kunnas in healthy individuals (treated with pravastatin). Individuals with the "a" allele of the *4b/4a* VNTR show a significant enhancement in vasodilation compared to individuals with the "bb" genotype, possibly due to a greater raise in endothelial NO production (102).

All these mechanisms seem to support the idea that statins are more useful in preventing cardiovascular diseases, by countering the mechanisms underlying the development of cardiovascular diseases, particularly in subjects with the CC genotype (1, 41).

Conclusion

Further studies are needed to elucidate the functional and clinical implications of eNOS3 polymorphism. The finding of biomarkers able of predicting a particular phenotype with accuracy is a challenge that will probably involve large scale studies, with results applicable in different populations.

Efforts should be oriented towards an evaluation of the effects of NOS3 genetic variants on biochemical parameters reflecting *eNOS* gene expression and enzymatic activity (in patients developing different pathologies). This approach can permit progress in our understanding of how eNOS genetics

may contribute to cardiovascular disease and help us optimize the relevant drug therapy.

We propose a change in treatment paradigm, from an approach based on average effects (reported in large cohorts) to individualized treatment to revolutionize both patients' therapy and manage adverse health effects, especially for cardiovascular disease patients.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

All the Authors contributed to the conception and design of the article and approved the final version submitted for publication.

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Received February 21, 2019
Revised June 9, 2019
Accepted June 19, 2019

STUDIA UBB CHEMIA, LXIV, 1, 2019 (p. 147-159)
(RECOMMENDED CITATION)
DOI:10.24193/subbchem.2019.1.12

A COMPARISON BETWEEN INSULIN RESISTANCE SCORES PARAMETERS IN IDENTIFYING PATIENTS WITH METABOLIC SYNDROME

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ABSTRACT As a consequence of the epidemic of obesity, prediction of metabolic syndrome (MetS) is relevant because of its subsequent association with type 2 diabetes and cardiovascular disease. Also, MetS is considered a state of insulin resistance (IR). This study aimed to evaluate the capacity of different indirect IR scores to identify patients with MetS, compared to standard criteria of MetS diagnosis. To the best of our knowledge, the cut-off values of these indexes, as indicators of MetS, in the Romanian population, have not been established. We used a non-parametric extension of the induced ROC regression methodology to determine the cut-off values of HOMA-IR, QUICKI, McAuley indexes, taking into account the MetS components instead of using 90th percentile criteria. Although HOMA is more commonly used in practice (probably due to its easy use), McAuley Score seems to have a better specificity in identifying patients with MetS. This is different between men and women; if in women, McAuley index has the highest sensitivity, in men, HOMA and QUICKI indexes have the highest sensitivity and specificity.

Keywords: *insulin, glycemia, HOMA, QUICKI, McAuley, metabolic syndrome*

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INTRODUCTION

Metabolic Syndrome (MetS) is associated with increased cardiovascular risk, being a common cause of atherosclerotic vascular disease. Also, MetS is considered a state of insulin resistance (IR).

Insulin resistance causes increased atherogenesis and atherosclerotic plaque instability by inducing proinflammatory activity in vascular and immune cells [1,2]. Insulin resistance enhances the risk of developing type 2 diabetes mellitus and is an independent risk factor for major cardiovascular events in patients with preexisting arterial disease [3,4]. Apart from influencing clinical disease development and mortality [5], IR is now recognized to play an important role in preclinical (silent) CAD. According to recent studies, IR is associated with asymptomatic myocardial perfusion defects in normotensive adults with preclinical diabetes [6] and is linked to angiographically documented silent CAD in patients with type 2 diabetes[7,8].

The quantification of IR can be achieved by evaluating peripheral insulin sensitivity in vivo with methods such as the hyperinsulinemic-euglycemic clamp technique or the intravenous glucose tolerance test. They are complicated, time-consuming and expensive methods, unsuitable for clinical studies. Simpler indirect methods have been promoted for quantification of IR. Moreover, several components of MetS (dysglycemia, abdominal obesity, dyslipidemia) are pathophysiologically related to IR.

This study aimed to evaluate the capacity of different indirect IR scores to identify patients with MetS, compared to standard criteria of MetS diagnosis [9]. The homeostatic model assessment of IR (HOMA-IR) is a mathematical model that predicts insulin sensitivity, measured by fasting blood glucose and basal insulin. It is strongly correlated with the hyperinsulinemic-euglycemic clamp (r=0.88), the gold standard for assessing insulin resistance (IR) [10]. It is also correlated positively with the components of MetS such as central obesity, lipid abnormalities and hypertension. However, there is great variability in the threshold HOMA-IR levels to define IR. Most studies have determined the cut-off values based on the percentile criteria (80th or 90th) of values in the general population.

Quantitative insulin sensitivity check index (QUICKI) can be determined from a fasting blood sample and correlates with glucose clamp measures of insulin sensitivity (r=0.78) [11]. QUICKI is similar to HOMA, except that QUICKI transforms the data by taking both the logarithm and the reciprocal of the glucose-insulin product, thus slightly skewing the distribution of fasting insulin values. Moreover, the two methods correlate well.

McAuley index is a score based on a weighted combination of insulin and triglycerides, corrected for weight, determined from a fasting blood sample [12].It is used for predicting IR in normoglycemic individuals.

IR probability score (IR-PS) is based on insulin, C-peptide, creatinine, TG/HDL-C, and BMI. It has been recently used to assess IR in apparently healthy individuals [13].

The use of these indexes in clinical practice is limited because of the absence of reference values for normal and impaired insulin sensitivity. HOMA-IR and QUIKI are suitable for clinical use, while McAuley index is suitable for epidemiological studies.

To the best of our knowledge, the cut-off values of these indexes, as indicators of MetS, in the Romanian population, have not been established. We used a non-parametric extension of the induced ROC regression methodology [14] to determine the cut-off values of HOMA-IR, QUICKI, McAuley indexes, taking into account the MetS components instead of using 90th percentile criteria. The purpose of this study was to evaluate the capacity of different IR scores to identify patients with MetS, compared to standard criteria of MetS diagnosis.

RESULTS AND DISCUSSION

The general characteristics of the studied group, including 84 subjects of which 21 men and 63 women with a mean age of 56.89±11.05 years, are shown in Table 1. Mean values for systolic and diastolic blood pressure, BMI, waist circumference, plasma leptin, total cholesterol, triglycerides, HDL cholesterol, fasting glucose, HbA1c and indirect insulin resistance indexes (HOMA, QUICKI, McAuley) are included in these data. Statistically significant differences between the patients with and without MetS were found for age, obesity, abdominal circumference, BMI, hypertension, diabetes, systolic blood pressure, diastolic blood pressure, triglycerides, insulin, HOMA, QUICKI, McAuley indexes.

Patients with MS presented higher insulin resistance (estimated through HOMA index 1.59 vs. 1.04, p=0.0001 and McAuley Score 2.37±0.43 vs. 1.89 ±0.36, p=0.0001) and lower insulin sensitivity (0.35±0.026 vs. 0.37±0.026, p=0.0001).

HOMA was significantly correlated with BMI (correlation coeff =0.372, p<0.001), SBP (correlation coeff =0.250, p=0.022), abdominal circumference (correlation coeff =0.464, p<0.001), glycemia (correlation coeff =0.592, p<0.001), the presence of diabetes (correlation coeff =0.403, p<0.001), serum TG (correlation coeff =0.432, p<0.001), the presence of MS (correlation coeff =0.443, p<0.001), and was inversely correlated with the HDL-cholesterol value (correlation coeff =-0.287, p=0.008)

Performing multivariate regression analysis for HOMA we have found that diabetes was the only independent predictors for HOMA (coefficient of determination = 3.393, p<0.001) (Table 2).

Table 1. – Characteristics of the studied group

		Total	Without MetS	MetS	p
Number	No (%)	84 (100)	29 (34.5)	55 (65.5)	0.0064
Age	Mean±SD	56.89±11.05	53.10 ±10.69	58.89±10.80	0.021
Sex	No (%)				
F		63 (75)	27 (93.10)	36 (65.45)	0.0118
M		21 (25)	2 (6.9)	19 (34.54)	
Smoking	No (%)				
Yes		21 (25)	11 (37.9)	10 (18.18)	0.08
No		63 (75)	18 (62.1)	45 (81.81)	
Obesity	No (%)				
Yes		32 (38.1)	6 (20.7)	26 (47.3)	0.0316
No		52 (61.9)	23 (79.3)	29 (52.7)	
AC	Mean±SD	96.57±12.42	89.55±11.60	100.27±11.27	0.0001
BMI	Mean±SD	28.81±4.22	26.89±4.42	29.82±3.78	0.0021
HTN	No (%)				
Yes		54 (64.3)	13 (44.8)	41 (74.6)	0.0138
No		30 (35.7)	16 (55.2)	14 (25.4)	
Diabetes	No (%)				
Yes		13 (15.5)	0 (0)	13 (23.6)	0.0114
No		71 (84.5)	29 (100)	42 (76.4)	
CVD	No (%)				
Yes		20 (23.8)	7 (24.1)	13 (23.6)	NS
No		64 (76.2)	22 (75.8)	42 (76.4)	
Glycemia	Mean±SD	97.35±16.76	86.75±8.30	102.94±17.43	< 0.0001
Total cholesterol	Mean±SD	218.09±43.58	221.41±35.86	216.34±47.36	Ns
LDL cholesterol	Mean±SD	139.82±37.05	148.13±30.30	135.43±39.71	Ns
HDL cholesterol	Mean±SD	47.15±8.5	51.68±6.93	44.76±8.32	0.0002
TG	Mean±SD	155.52±78.01	108.17±38.30	180.49±82.19	< 0.0001
SBP	Mean±SD	136.42±19.67	127.58±16.4	141.09±19.78	0.0023
DBP	Mean±SD	84.82±10.33	80.34±8.85	87.18±10.35	0.0034
Insulin*	Mean±SD (median)	7.58±5.18 (5.7)	6.22±4.05 (4.8)	8.29±5.59 (6.5)	0.0014
HOMA index *	Mean±SD (median)	1.86±1.47 (1.36)	1.34±0.91 (1.04)	2.14±1.63 (1.59)	0.0001
HOMA BETA*	Mean±SD (median)	88.61±56.36 (73.24)	98.59±60.73 (78.10)	83.52±53.86 (69.18)	NS
QUICKI index	Mean±SD	0.35±0.02	0.37±0.026	0.35±0.026	0.0001
Glycemia/insulin	Mean±SD	15.88±6.02	16.87±5.43	15.36±6.30	NS
McAuley index	Mean±SD	2.06±0.45	2.37±0.43	1.89±0.36	0.0001

*Not meeting the normality condition; for normal distribution data Student test was used; for not-normally distributed data Mann-Whitney test was used, for categorical data - $\chi 2$ test was used; AC – abdominal circumference, BMI- body mass index, HTN – hypertension, CVD-cardiovascular disease, TG – triglycerides, SBP - systolic blood pressure, DBP - diastolic blood pressure

Table 2. – Multivariate regression analysis for HOMA – model summary and coefficients

Model Summary					
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	a. Predictors: (Constant), SBP
1	.072[a]	.005	-.007	1.47549	b. Predictors: (Constant), SBP, AC
2	.227[b]	.052	.028	1.44949	c. Predictors: (Constant), SBP, AC, **DM_NO**
3	.467[c]	.218	.188	1.32472	d. Predictors: (Constant), SBP, AC, **DM_NO,** TG
4	.503[d]	.253	.215	1.30242	e. Predictors: (Constant), SBP, AC, **DM_NO**, TG, HDL
5	.505[e]	.255	.207	1.30924	f. Predictors: (Constant), SBP, AC, **DM_NO**, TG, HDL, **MS_NO**
6	.506[f]	.256	.198	1.31701	g. Dependent Variable: HOMA index

Coefficients[a]						
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.135	1.135		1.000	.320
	SBP	.005	.008	.072	.654	.515
2	(Constant)	-.552	1.400		-.394	.694
	SBP	-.002	.009	-.029	-.246	.806
	AC	.028	.014	.238	1.992	.050
3	**(Constant)**	.628	1.311		.479	.633
	SBP	-.006	.008	-.082	-.744	.459
	AC	.019	.013	.159	1.429	.157
	DM_NO	1.708	.415	.423	4.120	.000
4	(Constant)	.462	1.292		.358	.722
	SBP	-.006	.008	-.077	-.711	.479
	AC	.014	.013	.120	1.080	.283
	DM_NO	1.483	.424	.367	3.497	.001
	TG	.004	.002	.202	1.940	.056
5	(Constant)	.938	1.716		.547	.586
	SBP	-.005	.008	-.073	-.669	.506
	AC	.013	.013	.111	.978	.331
	DM_NO	1.497	.427	.370	3.502	.001
	TG	.004	.002	.187	1.675	.098
	HDL	-.008	.018	-.045	-.424	.672
6	(Constant)	.967	1.730		.559	.578
	SBP	-.006	.008	-.080	-.711	.479
	AC	.012	.014	.104	.897	.373
	DM_NO	1.477	.435	.366	3.393	.001
	TG	.003	.002	.178	1.531	.130
	HDL	-.006	.019	-.037	-.326	.745
	MS_NO	.110	.385	.036	.287	.775

SBP – systolic blood pressure, AC – abdominal circumference, DM_NO- presence/absence of diabetes, MS_ No - presence/absence of metabolic syndrome, TG – triglycerides, HDL-HDL-cholesterol

Table 3. – Multivariate regression analysis for QUICKI index – model summary and coefficients

Model Summary					
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	
1	.323[a]	.104	.093	.02740	a. Predictors: (Constant), BMI
2	.417[b]	.174	.153	.02648	b. Predictors: (Constant), BMI, **AC**
3	.536[c]	.288	.261	.02474	c. Predictors: (Constant), BMI, **AC**, **DM_NO**
4	.595[d]	.354	.322	.02370	d. Predictors: (Constant), BMI, **AC**, **DM_NO**, TG
5	.602[e]	.362	.321	.02371	e. Predictors: (Constant), BMI, **AC**, **DM_NO**, TG, HDL
6	.609[f]	.371	.322	.02370	f. Predictors: (Constant), BMI, **AC**, **DM_NO**, TG, HDL, **MS_NO** g. Dependent Variable: QUICKI index

Coefficients [a]						
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.422	.021		20.388	.000
	BMI	-.002	.001	-.323	-3.087	.003
2	(Constant)	.452	.023		19.685	.000
	BMI	.000	.001	.034	.198	.844
	AC	-.001	.000	-.443	-2.608	.011
3	(Constant)	.438	.022		20.104	.000
	BMI	-7.553E-005	.001	-.011	-.070	.945
	AC	-.001	.000	-.323	-1.990	.050
	DM_NO	-.028	.008	-.349	-3.583	.001
4	(Constant)	.442	.021		21.119	.000
	BMI	-8.645E-005	.001	-.013	-.083	.934
	AC	-.001	.000	-.271	-1.734	.087
	DM_NO	-.022	.008	-.274	-2.819	.006
	TG	.000	.000	-.277	-2.853	.006
5	(Constant)	.421	.030		14.131	.000
	BMI	-6.935E-005	.001	-.010	-.067	.947
	AC	-.001	.000	-.258	-1.642	.105
	DM_NO	-.022	.008	-.282	-2.892	.005
	TG	-8.977E-005	.000	-.243	-2.361	.021
	HDL	.000	.000	.095	.964	.338
6	(Constant)	.421	.030		14.142	.000
	BMI	-5.263E-005	.001	-.008	-.051	.960
	AC	-.001	.000	-.230	-1.440	.154
	DM_NO	-.021	.008	-.264	-2.664	.009
	TG	-7.924E-005	.000	-.215	-2.014	.047
	HDL	.000	.000	.068	.668	.506
	MS_NO	-.007	.007	-.116	-1.033	.305

BMI- body mass index, AC – abdominal circumference, DM_NO- presence/absence of diabetes, MS_ No- presence/absence of metabolic syndrome, TG – triglycerides, HDL- HDL-cholesterol

Table 4. – Multivariate regression analysis for McAuley index – model summary and coefficients

Model Summary					
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	a. Predictors: (Constant), BMI
1	.461[a]	.212	.203	.40513	b. Predictors: (Constant), BMI, **AC**
2	.501[b]	.251	.233	.39743	c. Predictors: (Constant), BMI, **AC**, **DM_NO**
3	.572[c]	.327	.302	.37915	d. Predictors: (Constant), BMI, **AC, DM_NO**, HDL
4	.616[d]	.379	.347	.36649	e. Predictors: (Constant), BMI, **AC**, **DM_NO**, HDL, **MS_NO**
5	.651[e]	.424	.387	.35515	f Dependent Variable: McAULEY score

Coefficients[a]						
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3.486	.306		11.381	.000
	BMI	-.049	.011	-.461	-4.699	.000
2	(Constant)	3.831	.344		11.127	.000
	BMI	-.021	.017	-.194	-1.199	.234
	AC	-.012	.006	-.332	-2.052	.043
3	(Constant)	3.653	.334		10.946	.000
	BMI	-.025	.017	-.230	-1.488	.141
	AC	-.009	.006	-.234	-1.483	.142
	DM_NO	-.355	.118	-.284	-3.000	.004
4	(Constant)	2.876	.442		6.507	.000
	BMI	-.024	.016	-.225	-1.501	.137
	AC	-.007	.006	-.186	-1.210	.230
	DM_NO	-.352	.114	-.282	-3.080	.003
	HDL	.013	.005	.234	2.573	.012
5	(Constant)	2.962	.430		6.893	.000
	BMI	-.024	.016	-.220	-1.518	.133
	AC	-.004	.006	-.116	-.763	.448
	DM_NO	-.281	.114	-.225	-2.456	.016
	HDL	.008	.005	.155	1.650	.103
	MS_NO	-.243	.098	-.256	-2.475	.015

AC – abdominal circumference, DM_NO- presence/absence of diabetes, MS_ NO - presence/absence of metabolic syndrome, TG – triglycerides, HDL- HDL-cholesterol

QUICKI was significantly negatively correlated with BMI (correlation coeff = -0.323, p=0.003), abdominal circumference (correlation coeff =-0.416, p<0.001), the presence of diabetes (correlation coeff = -0.429, p<0.001), glycemia (correlation coeff =-0.530, p<0.001), the TG value (correlation coeff =-0.433,

p=0.001), the presence of MS (correlation coeff =-0.417, p<0.001), and was directly correlated with HDL-cholesterol (correlation coeff =0.262, p=0.016) For QUICKI, the independent predictors factors were found to be – diabetes (coefficient of determination = -2.664, p=0.009) and triglycerides (coefficient of determination =-2.014, p=0.047) (Table 3).

McAuley index was inversely correlated with BMI (correlation coeff = -0.461, p<0.001), abdominal circumference (correlation coeff =-0.488, p<0.001), glycemia (correlation coeff =-0.392, p<0.001), the presence of DM (correlation coeff =-0.375, p<0.001), the serum TG value (correlation coeff =-0.775, p<0.001), the presence of MS (correlation coeff =-0.507 p<0.001), and was directly correlated with HDL (correlation coeff =0.337, p=0.002). For McAuley index, the independent predictors factors were found to be – diabetes (coefficient of determination = -2.225, p=0.016) and metabolic syndrome (coefficient of determination = -2.475, p=0.015) (Table 4).

The determined areas under the ROC curve were as follows: 0.769 for HOMA, 0.769 for QUICKI index, 0.818 for McAuley Score. Diagnostic cut-off levels with optimum sensitivity and specificity were found to be 1.07 for *HOMA* (sensitivity 87.27%, specificity 62.07%), 2.14 for *McAuley* Score (sensitivity 81.82%, specificity 75.86%), 0.37 for *QUICKI* index (sensitivity 87.27%, specificity 62.07%) (Table 5).

Table 5. Areas under the ROC for HOMA, QUICKI, McAULEY indexes

AUROC (95%CI)		**HOMA**	**QUICKI**	**McAuley**	**P***	**P****	**P*****
	Global	0.769 (0.664-0.854)	0.769 (0.664-.854)	0.818 (0.718-0.893)	NS	NS	NS
	Women	0.736 (0.610- 0.839)	0.736 (0.609 -0.839)	0.837 (0.723 - 0.918)	NS	0.034	0.04
	Men	0.947 (0.754 -0.992)	0.947 (0.754 -0.992)	0.737 (0.502 -0.901)	NS	NS	NS

P* - between HOMA and QUICKI; P** - between HOMA and McAuley;P*** - between QUICKI and McAuley

Subsequently, we calculated the cut-off values for the 3 IR indexes: HOMA, QUICKI and McAuley, globally (Figure 1), in women (Figure 2). Overall, the highest specificity was found for McAuley index (75.86%). By analyzing separately in women the cut-off values of the IR indexes, their

sensitivity and specificity, respectively, it was found that in women, McAuley index had the highest sensitivity (97.2%). It was not possible to performe a detailed analyses for men, because the number of males without MS was very small.

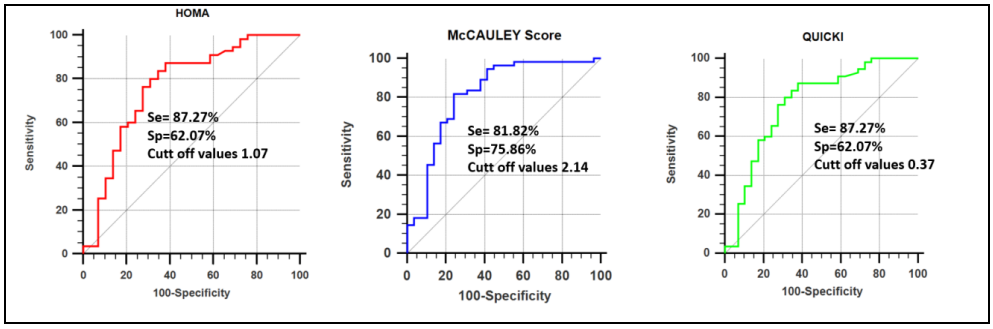

Figure 1. – AUROCs, Se, Sp and cut-off values - globally

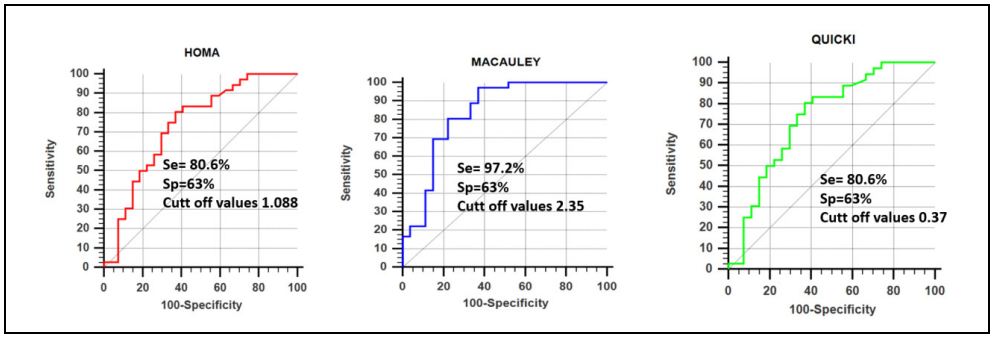

Figure 2. – AUROCs, Se, Sp and cut-off values in women

Although other authors [15] consider that the insulin resistance diagnosis is established at a HOMA index value >2.7 [16], in our study, the cut-off value for the diagnosis of MS was 1.07.

Cut-off values differ depending on the definition of MS; thus, Gayoso-Diz et al. [14] showed that using the IDF definition, like in our study, the ROC value was 0.69, and using the ATPIII definition, the ROC value was 0.72.

These results are similar to the study conducted by Esteghamati, who found an AUC of 0.65 (0.63, 0.67) for IDF and 0.68 (0.66, 0.70) for ATPIII [17]. In the current study, the ROC value was 1.07 for the HOMA index.

HOMA-IR is a reliable surrogate method to estimate IR in an epidemiological or clinical setting. However, its threshold levels vary widely; the cut-off values of HOMA-IR are usually defined by population-based percentile criteria. Moreover, these cut-off values are different depending on ethnicity, clinical methods of estimation, and metabolic conditions of the studied populations [18,19].

In the Spanish population, the threshold value of HOMA-IR decreases from 3.46 using 90th percentile criteria [20] to 2.05 taking into account the MetS components. Our HOMA-IR cut-off levels are relatively low compared to those reported in a study on healthy Italian patients [21], with a value of 2.77, and in a Spanish non-diabetic population [22], with a value of 3.8.

Gayoso-Diz et al. [14] concluded that the effect of age and gender on the ability of HOMA-IR to identify subjects with a cardiometabolic risk phenotype should be taken into account when estimating its values in different populations.

Shalaurova [23] showed in a study that the IR score was strongly correlated with triglycerides (r = 0.74) and fasting insulin levels (r = 0.51), and was inversely correlated with HDL-C (r = -0.67), like in the current study, where the best correlation with TG was found for McAuley index.

Tosi et al. [24] reported that the insulin resistance indexes HOMA and QUICKI had a sensitivity of 50.9 and 57.7, respectively, in identifying insulin resistance, significantly lower compared to the current study, while their specificity was 88.3, and 86.2, respectively, AUC for HOMA and QUICKI being 0.798. In our study, the specificity of these indexes in identifying patients with MS was lower, and AUC was higher. The authors of this study concluded that these IR indexes were very well correlated with the hyperinsulinemic-euglycemic clamp, which is the gold standard in IR diagnosis. McAuley index has the highest sensitivity, in women. An important element in the current study is the fact that independent predictors for the insulin resistance indexes are different: for HOMA: diabetes, for QUICKI: diabetes and triglycerides and for McAuley: diabetes and metabolic syndrome.

CONCLUSIONS

The measurement of IR remains a challenge. The current gold standard test is the hyperinsulinemic-euglycemic clamp technique, in which a constant rate of insulin infusion is balanced with concomitant variable glucose infusion to maintain euglycemia; it is an invasive time- and labor-intensive approach - not suitable for epidemiological and diagnostic studies. Although HOMA is more commonly used in practice (probably due to its easy use), McAuley Score seems to have a better specificity in identifying patients with MetS. In women, McAuley index has the highest sensitivity.

EXPERIMENTAL SECTION

The study was carried out at the 4th Department of Internal Medicine, Cluj-Napoca; The rights of the patients regarding the confidentiality of personal information were respected in agreement to Helsinki declaration of Ethical Principles for Medical Research Involving Human Subjects.

84 consecutive participants completed a questionnaire regarding their personal and family medical history. All were subjected to a complete physical exam.

For each patient, weight, height and abdominal circumference (midway between the inferior margin of the last rib and the iliac crest in horizontal plane while in upright position) were measured. The body mass index (BMI) was calculated (weight (kg)/[height (m)]2); subjects with a BMI ≥ 30 kg/m2 were considered obese.

Blood samples were collected after an overnight fast of >8 h. Plasma glucose levels were measured using a hexokinase enzymatic reference method. Fasting insulin levels were measured using a radioimmunoassay method or ELISA. Fasting lipids were analyzed, and for the present study, serum levels of cholesterol ≥5.172 mmol/L and triglycerides ≥1.7 mmol/L were considered abnormal.

The diagnosis of MetS was made based on International Diabetes Federation criteria, including the presence of abdominal obesity (waist circumference ≥94 cm for men and ≥80 cm for women) plus at least two of the following criteria: 1) fasting glucose ≥100 mg/dL; 2) systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg or treatment of previously diagnosed hypertension; 3) HDL-cholesterol <40 mg/dL for men and <50 mg/dL for women or treatment with high-dose omega-3 therapy; 4) triglycerides ≥150 mg/dL or drug treatment with fenofibrate.

Insulin resistance was assessed by:

- HOMA-IR = insulin (μU/mL) * glycemia (mg/dl) / 405;
- QUICKI =1 / [log (insulin (μU/mL)) + log (glycemia)];
- McAuley = exp [3.29-0.25 * log (insulin) - 0.22 * log (BMI) – 0.28 * log (triglyceride)];

The blood insulin level can be expresed in international units, such as μIU/mL or in molar concentration, a typical blood level between meals is 8–11 μIU/mL (57–79 pmol/L). Statistical analysis – *the* data were analyzed using SPSS 16.0 for Windows and MedCalc 10.3.0.0 software programs. Descriptive statistics was performed. Data's normality was assessed using Kolmogorov-Smirnov test. Numerical data were compared using Student or Mann-Whitney test; for categorical variable $\chi 2$ test was used. Univariate and

multivariate analysis was performed. Cut-off values, optimum sensitivity, specificity and area under the receiver operating characteristic (ROC) curve were evaluated. $p < 0.05$ was considered significantly statistic.

ACKNOWLEDGMENTS

Authors greatly acknowledge the financial support offered by GRANT ID 2246.

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DRUG METABOLISM REVIEWS
https://doi.org/10.1080/03602532.2019.1656226

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REVIEW ARTICLE

Clinical and genetic predictors of diabetes drug's response

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ABSTRACT

Diabetes is a major health problem worldwide. Glycemic control is the main goal in the management of type 2 diabetes. While many anti-diabetic drugs and guidelines are available, almost half of diabetic patients do not reach their treatment goal and develop complications. The glucose-lowering response to anti-diabetic drug differs significantly between individuals. Relatively little is known about the factors that might underlie this response. The identification of predictors of response to anti-diabetic drugs is essential for treatment personalization. Unfortunately, the evidence on predictors of drugs response in type 2 diabetes is scarce. Only a few trials were designed for specific groups of patients (e.g. patients with renal impairment or older patients), while subgroup analyses of larger trials are frequently unreported. Physicians need help in picking the drug which provides the maximal benefit, with minimal side effects, in the right dose, for a specific patient, using an omics-based approach besides the phenotypic characteristics.

ARTICLE HISTORY
Received 6 February 2019
Accepted 9 August 2019

KEYWORDS
Personalized diabetes therapy; drug response; drug metabolism; omics-based therapy; type 2 diabetes

Introduction

According to The International Diabetes Federation, 425 million adults had diabetes in 2017, and the number is expected to reach 629 million in 2045 (IDF 2017).

Type 2 diabetes (T2D) is a chronic disease characterized by decreased insulin secretion and increased insulin resistance. It accounts for more than 90% diabetes cases. T2D is caused by the interplay of several genes and environmental factors.

Candidate gene association studies, as well as genome-wide association studies (GWASs), have identified over 100 common genetic variants associated with T2D or glycemic traits. However, they only explain a small fraction of the heritability of the disease, suggesting that most genes involved in T2D risk have not yet been identified.

Glycemic control is the major goal in the management of T2D. While many anti-diabetic drugs are available, almost half of diabetic patients do not reach their treatment goal.

The American Diabetes Association (ADA) recommend a patient-centered approach (ADA 2019). The drug should match the patient's characteristics: magnitude of glycemic imbalance, history of cardiovascular disease, impact on weight, risk of hypoglycemia, renal function, administration route (oral or injectable), potential side effects, cost, and patient preference. Lifestyle optimization and metformin are recommended as first-line therapy by most organizations (UK National Institute for Health and Care Excellence [NICE] 2017; [DC] Diabetes Canada Clinical Practice Guidelines 2018; ADA 2019). Several antidiabetic drugs are available after metformin failure [sulfonylureas (SUs), meglitinides, thiazolidinediones (TZDs), GLP-1-receptor agonists, DPP-4 inhibitors, SGLT2 inhibitors, α-glucosidase inhibitors (α-GI) and insulins]. While routine practice and clinical trials show that the glucose-lowering response to any drug differ between individuals, relatively little is known about the differential efficacy of diabetes drugs and the factors that might underlie this early response. This heterogeneity can be the effect of genetic and non-genetic factors. The results of major diabetes trials showed that hemoglobin A1c (HbA1c) levels tend to increase steadily over many years despite an initial sharp decline after the initiation of oral drug treatment. HbA1c is an indirect marker for average blood glucose

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levels over the previous 3 months. This phenomenon is described as the 'secondary ineffectiveness' or 'monotherapy failure.' Cumulative incidence of monotherapy failure at 5 years was 34% with glyburide (SUs), 21% with metformin and 15% with rosiglitazone (TZDs) (Kahn et al. 2006).

The identification of predictors of response to glucose-lowering drugs, in the early phase but also in long term is essential for appropriate treatment personalization. Physicians need help in choosing 'the right drug for the right patient at the right time.' Pharmacogenomics has showed that polymorphisms in genes involved in drug absorption, transport, metabolism, and action could affect the pharmacokinetics (PK) or pharmacodynamics (PD) of drugs. Despite an increasing number of potentially actionable pharmacogenetic variants and freely available guidelines, the use of pharmacogenetic data to predict an individual's drug response is minimal to moderate (Fodor and Karnieli 2016). Although in their infancy, additional 'omics', like microbiota, epigenomic and metabolomic are expected to provide further drug-response markers (Figure 1).

Metformin therapy

Metformin is recommended as first-line therapy in T2D by most guidelines (NICE 2017; DC 2018; ADA 2019) because of its efficacy, safety (low risk of hypoglycemia, absence of weight gain), low cost, and cardiovascular benefit. The patients' response to metformin is highly variable, ranging from no response to improvement in HbA1c up to 4%, while 35% of patients fail to achieve initial glycemic control on metformin monotherapy (Cook et al. 2007). ADOPT study group also demonstrated the progressive decline of metformin effect over time (Kahn et al. 2006). Metformin works as an insulin sensitizer, leading to suppression of hepatic glucose production and increased glucose uptake in muscle. It is used for more than 60 years, but the mechanisms by which it increases insulin sensitivity are still uncertain. The activation of AMP-activated protein kinase has been thought to be responsible for the suppression of hepatic glucose production. The genetic variants of AMPK subunits have been associated with metformin response (Jablonski et al. 2010). Beyond its primary role in decreasing hepatic glucose production, metformin is uptaken by the muscle cells via organic cation transporter 3 (OCT3) and increases glucose uptake via translocation of glucose transporter 4 (Chen et al. 2010).

Several solute carrier (SLC) transporters are responsible for the intestinal absorption of metformin from the lumen, including plasma membrane monoamine transporter (PMAT/ENT4) and organic cation transporter 3 (OCT3), while the transport of metformin into the bloodstream involves organic cation transporter 1 (OCT1) (Pawlyk et al. 2014).

Then metformin is up taken in target tissues by other members of the OCT family. OCT1 is located mainly at the sinusoidal cells of the liver while OCT2 at the renal tubular cells, being responsible for the hepatic (OCT1)

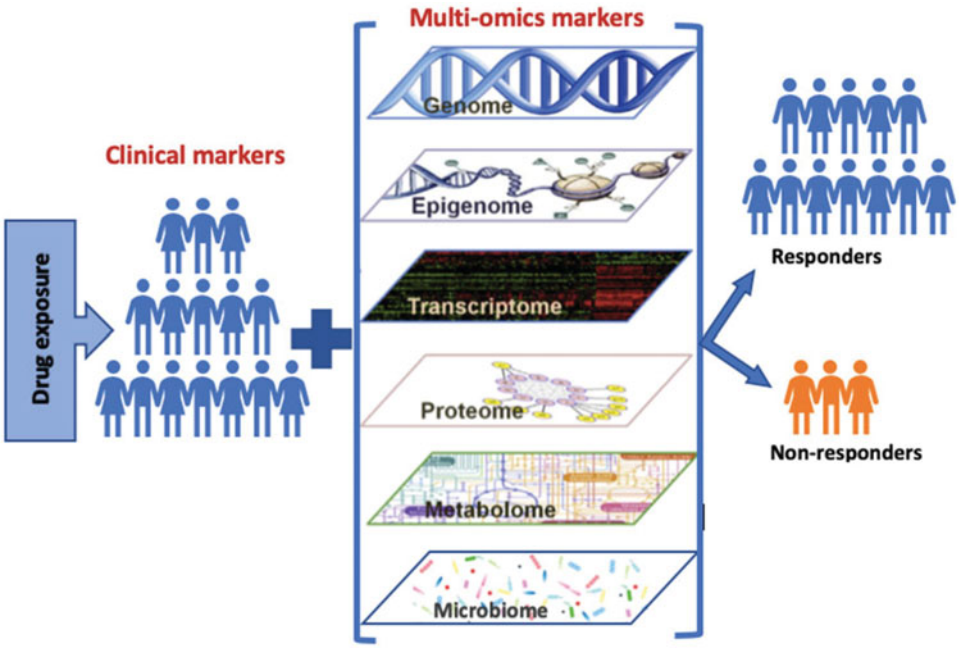

Figure 1. Predictors of diabetes drug response (Adapted from Sun and Hu 2016).

and renal (OCT2) uptake of metformin from the blood. OCT3 is in muscle cells. Metformin circulates unbound to plasma proteins and is cleared by the kidney as unchanged drug. The tubular secretion has a significant role in metformin elimination, as its renal clearance is much greater than glomerular filtration rate.

Metformin is removed from target tissues by multi-antimicrobial drug and toxin extrusion transporters (MATE). Thus, MATE1 contribute to the efflux of metformin into biliary duct, whereas MATE1 and MATE2 contribute to the clearance of metformin into the urine (Pawlyk et al. 2014).

A systematic review up to 2012 (four cohort studies and six clinical trials) showed that there is limited data on the predictors of response to metformin and SUs at the beginning of therapy (Martono et al. 2015). Baseline HbA1c, older age, shorter disease duration and lower body mass index (BMI) were predictors of greater response to metformin.

A recent analysis of 698 Chinese patients with newly diagnosed T2D, randomized on metformin or acarbose (an α-glucosidase inhibitor) have shown that after 24 weeks of monotherapy, almost 80% of participants in both groups achieved HbA1c $< 7\%$. Lower fasting plasma glucose (FPG) and higher BMI were predictors of better response to metformin, while higher phase I insulin secretion and shorter duration of T2D were predictors of a better response to acarbose (Han J et al. 2017).

Another recent study identified predictors of long-term metformin response in 1056 T2D patients (Goswami et al. 2016). Higher plasma creatinine level was a significant predictor of greater metformin response. The model predicted that a 0.7 mg increase in creatinine level (from 0.6 to 1.3) will result in 20% increase in metformin exposure and a greater decrease in HbA1c (0.77% vs 0.96%). Additionally, bodyweight was inversely correlated with metformin response. The model estimated a decrease in response to metformin of 6% for an increase in body weight of 10 kg. Age was negatively correlated with disease progression and HbA1c. Thus, a 15 years' age difference (between 49 and 64 years) results in a change in HbA1c between 0.76% and 0.84% (Goswami et al. 2016).

The glycemic response to metformin is probably determined by the interaction of genetic and environmental factors. Clinical parameters such as BMI, age, diabetes duration, serum creatinine, baseline HbA1c only account for part of the variation. Genome-wide complex trait analysis of more than 2000 T2D patients, revealed that genetic variation contributes up to 34% of differential glycemic response to metformin (Zhou K et al. 2014). So far, only a few polymorphisms in genes affecting metformin response have been identified. Enhanced GWAS analyses, with adequate statistical power, are expected to find more genetic variants that would enable better metformin response.

Pharmacogenomic studies of several metformin transporter genes that influence metformin PK and drug efficacy have been reported (Table 1).

OCT1 (*SLC22A1*) is the major hepatic uptake transporter of metformin from the blood into hepatic sinusoidal cells. OCT1 has been identified also in the kidney and intestine.

Several *SLC22A1* polymorphisms have been found in cellular studies to exhibit reduced transport of metformin (Shu et al. 2007). Two of these *SLC22A1* polymorphisms are very common in Europeans (420del (rs72552763) has allele frequencies of 19% and R61C (rs12208357) of 7.2%) (Shu et al. 2007).

The results of *SLC22A1* reduced-function alleles on metformin PK have been contradictory. In a study of Shu et al., on 20 healthy volunteers, patients having one or more *SLC22A1* reduced-function variants (R61C, G401S, 420del, and G465R) had a lower renal clearance of metformin and slightly higher metformin plasma concentrations than patients with wild-type *SLC22A1* (Shu et al. 2008). In a parallel study, in 103 healthy Caucasians, Tzvetkov et al. showed an increased renal clearance of metformin in carriers of inactive *SLC22A1* alleles ($p = 0.04$), with slightly lower plasma metformin concentrations in the homozygous carriers of inactive *SLC22A1* alleles (Tzvetkov et al. 2009). They have shown a substantial inter-individual variability (3.8-fold) in the renal clearance of metformin, that was dependent on creatinine clearance and *SLC22A1* polymorphisms. While the creatinine clearance variance accounted for 42% of the variability in the renal clearance of metformin, the *SLC22A1* polymorphisms accounted for an additional 10%. The combined effect of the four amino acid variants (R61C, G401S, 420del, and G465R) was associated with an increase in the renal clearance of metformin by ∼20%. It has been suggested that the location of *SLC22A1* in the distal tubules is responsible for metformin reabsorption, explaining the increased renal elimination of metformin (Tzvetkov et al. 2009). Duong et al. reported no significant effect of reduced function *SLC22A1* allele (R61C, G401S, 420del or G465R) on the PK of metformin in 103 T2D patients (Duong et al. 2013). It is worth mentioning that most patients had one low activity variant of the *SLC22A1* gene and only 2 carriers had two low activity variants of *SLC22A1*. Similarly, Christensen et al., reported a huge (80-fold) inter-individual variability in trough steady-state metformin concentration in 159 DM2 patients, with an overall

Table 1. Polymorphisms commonly associated with metformin responsiveness in T2D.

Gene (protein)	Polymorphism	Drug effect	No. of cases	Reference
SLC22A1 (OCT1)	rs12208357 rs72552763 rs34130495 rs34059508	Decreased metformin response on glucose tolerance tests	20	(Shu et al. 2007)
	rs12208357	Increased metformin response (OR 1.56) in treatment-naive patients	1531	(Zhou K et al. 2009)
	rs72552763	No effect on metformin response		
	rs12208357 rs72552763 rs34130495 rs34059508	No effect on metformin response	253	(Choi et al. 2011)
	rs622342	Decreased metformin response by 0.28% HbA1c for each minor C allele	102	(Becker et al. 2009b)
	rs622342	No effect on metformin response;	990	(Jablonski et al. 2010)
	rs683369	Decrease the ability of metformin to prevent diabetes by 31% for each minor G allele		
	rs622342	No effect on metformin response	148	(Tkac et al. 2013)
	rs12208357 rs72552763 rs34130495 rs34059508 rs55918055	Increased metformin side effects, twice in individuals with 2 reduced-function OCT1 alleles	1940	(Dujic et al. 2015)
	rs34130495	Decreased metformin response by 0,7% HbA1c, for each allele	159	(Christensen et al. 2015)
	rs461473	Decreased metformin response by 1,2% HbA1c, per two copies of allele		
	rs622342	Decreased metformin response by 0,7% HbA1c, per two copies of allele		
	rs12208357 rs72552763 rs34130495 rs683369	No effect on metformin response	28	(Sam et al. 2017)
	rs12208357	No effect on metformin response (meta-analysis)	4557	(Dujic et al. 2017)
	rs72552763		4423	
	rs622342		5436	
SLC22A2 (OCT2)	rs316019	No effect on metformin effect	990	(Jablonski et al. 2010)
	rs662301	Decrease de ability of metformin to prevent diabetes by 43% for each minor allele T		
	rs316019	No effect on metformin effect	253	(Choi et al. 2011)
	rs316019	No effect on metformin effect	148	(Tkac et al. 2013)
	rs316019	No effect on metformin effect	145	(Stocker et al. 2013)
	rs316019	Increased metformin response by 1.1% HbA1C for each minor T allele	220	(Hou et al. 2015)
	rs316009	Increased metformin response (explains 8% variability in metformin response)	1056	(Goswami et al. 2016)
	rs8192675	Increased metformin response: 0.15% greater HbA1c reduction	13123	(Zhou K et al. 2016)
	rs316019	No effect on metformin response (meta-analysis)	5224	(Dujic et al. 2017)
SLC47A1 (MATE1)	rs2289669	Increased metformin response by 0.3% HbA1C for each minor A allele	116	(Becker et al. 2009a)
	rs2289669	Increased metformin response for AA homozygotes vs G carriers by 0.55% HbA1C	148	(Tkac et al. 2013)
	rs2289669	Increased metformin response for AA homozygotes vs G carriers ($p = 0.055$)	189	(Choi et al. 2011)
	rs8065082	Increased the ability of metformin to prevent diabetes by 22% for each minor T allele and 42% for TT genotypes	990	(Jablonski et al. 2010)
	rs2252281	Increased metformin response: 15% greater relative reduction in Hb1Ac in CC-variant allele vs. all others	145	(Stocker et al. 2013)
	rs2289669	No effect on metformin response (meta-analysis)	5205	(Dujic et al. 2017)
	rs2252281		2651	
SLC47A2 (MATE2)	rs12943590	Reduced response to metformin: 12.3% lower relative reduction in HbA1c in AA-variant allele vs. all others	189	(Choi et al. 2011)
	rs12943590	Reduced response to metformin: the glucose AUC in homozygous AA (333 ± 37 mg/dL/h) vs. (295 ± 44 mg/dL/h; $p = 0.02$) in all others	57	(Stocker et al. 2013)
	rs34399035	Decreased metformin response by 0.8% HbA1c, for each allele	159	(Christensen et al. 2015)
	rs12943590	No effect on metformin response (meta-analysis)	4398	(Dujic et al. 2017)
ATM	rs11212617	Increased metformin response by 0.11% HbaA1c for each C allele and 0.61% for two alleles	3920	(Zhou K et al. 2011)
	rs11212617	Increased the ability of metformin to achieve HbA1c values $\leq$7% with an OR 1.24 (95% CI 1.04, 1.49, $p = 0.016$)	1365	(van Leeuwen et al. 2012)
	rs11212617	Increased the ability of metformin to reduce HbA1c (AA: 0.67 %, AC: 0.78 %, CC: 1.59)	274	(Zhou Y et al. 2014)
	rs11212617	No preventive effect of metformin in C carriers; HR = 1.17 compared with A carriers (95% CI 0.96–1.42, $p = 0.13$)	2994	(Florez et al. 2012)
STK11 PRKAA1 PRKAA2 PRKAB2 CAPN10	rs741765 rs249429 rs9803799 rs6690158 rs3792269	Increased the ability of metformin to prevent diabetes	990	(Jablonski et al. 2010)
			148	(Tkáč et al. 2015)

(*continued*)

Table 1. Continued.

Gene (protein)	Polymorphism	Drug effect	No. of cases	Reference
PRKAA1	rs249429	No significant effect Reduced metformin response by 0.26% HbA1c for		
CAPN10	rs3792267	each minor G allele		
SP	rs784892	Increased metformin response: 1.1% lower HbA1c in GG-variant	440	(Goswami et al. 2014)
	rs784888	(rs784892) that AA-reference		
	rs10747673			
	rs2683511			
	rs2694855			
AP2	chr13:74559166:D	Reduced metformin response	440	(Goswami et al. 2014)
KCNK16	rs2815022	Reduced metformin response	1056	(Goswami et al. 2016)
VPS13C	rs12907856	Increased metformin response (explains 6% variability in metformin response)	1056	(Goswami et al. 2016)
CSMD1	rs2954625	Reduced metformin response (explains 5% variability in	1056	(Goswami et al. 2016)
	rs2617102	metformin response)		
WWOX	rs7500549	Increased metformin response	1056	(Goswami et al. 2016)
FOXN3	rs7159552	Increased metformin response	1056	(Goswami et al. 2016)
EMILIN2	rs642887	Increased metformin response	1056	(Goswami et al. 2016)

trend to lower plasma concentrations in carriers of reduced function alleles in *SLC22A1* (none, one or two: 642, 542, 397 ng/ml; $p = 0.001$)(Christensen et al. 2011). Sixty-five patients were heterozygotes, carrying one low activity variant of *SLC22A1* (R61C, G401S, M420del, G465R), while only 10 patients carried two alleles.

Overall the effect of *SLC22A1* polymorphism on metformin PK is small and more significant in homozygous carriers, that are quite infrequent 3.8% (Tzvetkov et al. 2009), 1.94% (Duong et al. 2013), 6.28% (Christensen et al. 2011).

Intriguing results were reported on the effect of common variants in *SLC22A1* on glucose-lowering effect of metformin. Preliminary study by Shu et al., showed that healthy carriers of any one of the four decreased-function polymorphisms in *SLC22A1* (R61C, G401S, 420del, and G465R) have decreased metformin response during an oral glucose tolerance test (Shu et al. 2007). While Zhou et al., assessed the effect of two of these *SLC22A1* variants on metformin response in a large cohort of patients with T2D from Genetics of Diabetes Audit and Research Tayside Study (GoDARTS) found no significant effect on glucose-lowering effect of metformin, except for a treatment-naive subgroup of patients, carriers of the R61C genotype, who have better treatment response (OR 1.56; $p = 0.036$) (Zhou K et al. 2009). Finally, Shu's results have not been confirmed by the same group of researchers, stating that *SLC22A1* reduced-function variants (R61C, 420Del, G401S, and G465R) may not have a significant effect on metformin response in patients with T2DM (Choi et al. 2011). Becker et al. assessed whether *SLC22A1* gene polymorphisms are associated with metformin response in the Rotterdam Study. Out of 12 studied *SLC22A1* variants, different from those used by Shu et al, only the minor allele rs622342 was associated with metformin response (allele frequency of 27% in Caucasians) (Becker et al. 2009b). A large-scale genetic association study in the Diabetes Prevention Program (DPP) found a missense SNP in *SLC22A1* (rs683369, encoding Phe160Leu) as being protective from diabetes in the metformin users (HR 0.69, 95% CI 0.53–0.89, $p = 0.004$) (Jablonski et al. 2010). The authors interpreted this finding less robust, taking in account that this variation was one of the least functional human variant in Shu's study (Shu et al. 2007) and it was in weak LD with rs622342, the *SLC22A1* variant associated with metformin response in Rotterdam study (Becker et al. 2009b). Recently, Sam et al., identified 15 SNPs in *SLC22A1* gene (13 previously described) in 10 subjects, out of 28 children with severe obesity and insulin resistance. *SLC22A1* genotypes with presumably low activity did not significantly affect metformin PK or PD (HbA1c, HOMAIR) compared with *SLC22A1* wild type subjects (Sam et al. 2017). Christensen et al. reported lower plasma concentrations and decreased response to metformin in carriers of two variants *SLC22A1* rs622342 (described in Rotterdam Study), two alleles of *SLC22A1* rs461473, and one allele of *SLC22A1* rs34130495 (Christensen et al. 2011).

Finally, a meta-analysis across the cohorts of Metformin Genetics Consortium (MetGen) (including GoDARTDS, Rotterdam studies) in up to 7968 participants, showed no significant effect of three of these *SLC22A1* polymorphisms (rs12208357, rs72552763, rs622342) (Dujic et al. 2017).

It has been shown a significant effect of *SLC22A1* variance on metformin intolerance. The patients

Table 2. Polymorphisms commonly associated with SUs responsiveness in T2D.

Gene	Polymorphism	Drug effect	No. of cases	Reference
CYP2C9	rs1799853 rs1057910	Increased SUs response by 0.5% HbA1c per two copies of allele	1073	(Zhou K et al. 2010)
	rs1799853 rs1057910	Increased response to glibenclamide: 50% of variant carriers achieved plasma glucose <110 mg/dl compared with 25% in normal genotypes	80	(Surendiran et al. 2011)
	rs1057910	Increased response to gliclazide: 51.4% of variant carriers achieved HbA1c < 7.0 mmol/L compared with 32.2% in normal genotypes	746	(Zeng et al. 2016)
TCF7L2	rs12255372	Reduced SUs response, with OR for failure of 1.95	579	(Pearson et al. 2007)
	rs7903146	Reduced SUs response, with OR for failure of 1.73		
	rs7903146	Reduced SUs response, with OR for failure of 1.57	189	(Holstein et al. 2011)
	rs7903146	Reduced SUs response by 0.3% HbA1c	87	(Schroner et al. 2011b)
	rs12255372	Reduced SUs response, 19% increased treatment failure	250	(Dhawan and Padh 2016)
	rs7903146	Non-significant effect		
	rs4506565	Reduced SUs response, increased treatment failure		
	rs7903146	Increased response to glipizide: a faster and a steeper slope to glucose trough, on oral glucose tolerance test	608	(Srinivasan et al. 2018)
KCNQ1	rs163184	Decreased SUs response: 0.54 mmol/l lower reduction in FPG in GG-risk genotype compared with all others	87	(Schroner et al. 2011a)
	rs2237892	Increased gliclazide response: 2.53-fold decrease in HbA1c	91	(Li et al. 2017)
	rs2237895	Increased gliclazide response: 2.36-fold decrease in HbA1c		
	rs2237897 rs2237892	Increased SUs response: 0.7 mmol/l greater reduction in FPG in variant allele compared with CC wild type No effect on glibenclamide response	443	(Duan et al. 2016)
	rs12720449 rs2237892	No effect on glibenclamide response	747	(Ren et al. 2014)
KCNJ11	rs5219	Decreased glibenclamide response, OR for failure of 1.69	525	(Sesti et al. 2006)
	rs5219	Increased SUs response by 0.16% HbA1c per each allele	101	(Javorsky et al. 2012)
	rs5219	No effect on SUs response	228	(Nikolac et al. 2009)
	rs5219	No effect on SUs response	156	(Klen et al. 2014)
	rs5219	Decreased glibenclamide response, with increased risk for failure	209	(Phani et al. 2017)
	rs5210	Increased gliclazide response	661	(Feng et al. 2008)
	rs5219	Decreased SUs response, with increased risk for failure	278	(Shimajiri et al. 2013)
	rs5219	Decreased SUs response, with OR for failure of 1.65	100	(El-Sisi et al. 2011)
ABCC8	rs757110	Increased gliclazide response by 0.84% HbA1c	115	(Zhang et al. 2007)
	rs757110	Increased gliclazide response in risk GG-allele carriers by 0.3% HbA1c, compared with TT genotype	1268	(Feng et al. 2008)
	rs1799854	Decreased SUs response in risk TT genotype by 1.2% HbA1c compared with wild-type CC genotypes	228	(Nikolac et al. 2009)
	rs1799859	Increased SUs response in risk AA genotypes by 1.5% HbA1c compare with GG carriers	228	(Nikolac et al. 2009)
IRS-1	rs1801278	Reduced SUs response, with OR for failure of 2.1	477	(Sesti et al. 2004)
	rs1801278	Reduced SUs response, with OR for failure of 1.75	100	(El-Sisi et al. 2011)
	rs1801278	No effect on gliclazide response	661	(Feng et al. 2008)

carrying two *SLC22A1* reduced-function polymorphisms (nearly 8% of white Europeans) had increase twice the risk of metformin intolerance compared with those having wild type *SLC22A1* (Dujic et al. 2015). Moreover, there are several drugs that inhibit OCT1 transport and increase fourfold the risk of metformin intolerance in patients with reduced-function *SLC22A1* polymorphism. Lowering the metformin dose or a slow-release formulation would be helpful in patients who carry the risk variants (Dujic et al. 2015).

Since no association has been robustly replicated, further studies are needed to clarify the effect of *SLC22A1* genotype on metformin response. The overall effect of *SLC22A1* variations on metformin response seems to be small and significant in carriers of two *SLC22A1* reduced-function variants.

OCT2 *(SLC22A2)* is primarily expressed in distal renal tubules, leading to metformin uptake from circulation into renal epithelial cells. Loss of OCT2 function is expected to increase metformin plasma concentration and increase its glucose-lowering effect.

Healthy volunteers of European and African ancestries, carriers of one of the most common variant in *SLC22A2* (808 G/T, rs316019) (10% allele frequency) had a greater renal clearance of metformin, concomitant with lower metformin concentrations, comparing with homozygous for the reference *SLC22A2* (GG) (Chen et al. 2009). However, two other studies showed that metformin renal clearance was reduced in individuals of Asian ancestries who carried the 808 G/T allele (Song et al. 2008; Wang et al. 2008), while Tzvetko et al. have not seen any significant association of 14 genetic polymorphisms in OCT2 (including 808 G/T) with the renal

Table 3. Polymorphisms commonly associated with glucose-lowering effect of incretins in T2D.

Gene	Polymorphism	Drug effect	No. of cases	Reference
CTRB1/2	rs7202877	Reduced DPP-4i response by 0.5% HbA1c	354	(t' Hart et al. 2013)
TCF7L2	rs7903146	Reduce linagliptine response in TT compared with CC patients by ~0.26% HbA1c	961	(Zimdahl et al. 2014)
GLP1R	rs3765467	Increased DPP-4i response by 0.4% HbA1c	246	(Han E et al. 2016)
	rs6923761	Reduced DPP-4i response by 0.68% HbA1c in AA-variant carriers compared with wild type-G carriers	140	(Javorsky et al. 2016)
	rs6923761	Increased liraglutide response by 0.33% HbA1c (ns)	90	(de Luis et al. 2015)
	rs3765467	Decreased exenatide response	36	(Lin et al. 2015)
	rs761386	Increased exenatide response		
CDKAL1	rs7754840	Increased DPP-4i response by 0.1% HbA1c per allele	512	(Osada et al. 2016)
	rs7756992	Increased DPP-4i response by 0.13% HbA1c per allele		
KCNJ11	rs2285676	Reduced DPP-4i response, OR = 2.1	662	(Jamaluddin et al. 2016)
KCNQ1	rs163184	Reduced DPP-4i response, by 0.3% HbA1c for each risk-G allele	137	(Gotthardova et al. 2017)
PRKD1	rs57803087	Increased DPP-4i response	249	(Liao et al. 2017)
DPP4	rs2909451	Increased DPP-4 activity, OR = 1.76 in TT vs. CC	65	(Wilson et al. 2017)
	rs759717	Increased DPP-4 activity, OR = 1.9 in CC vs. GG		

elimination of metformin (Tzvetkov et al. 2009). Four different studies, including DPP, could not find any significant effect of this polymorphism on the glucose-lowering effect of metformin (Jablonski et al. 2010; Choi et al. 2011; Stocker et al. 2013; Tkac et al. 2013). Finally, the large meta-analysis of MetGen cohorts could not confirm the effect of rs316019 polymorphism on metformin response (Dujic et al. 2017). However, two more studies did find some effect. In a study of 220 newly diagnosed T2D Chinese patients treated with metformin for 1 year, heterozygous variant genotype (808 G/T) had a greater decrease in HbA1c comparing with the wild-type homozygote (−2.2% vs. −1.1%, $p < 0.05$) after adjustment for baseline HbA1c levels (Hou et al. 2015). They showed significant increase in metformin plasma concentration with decreased renal clearance. Goswami et al described a *SLC22A2* (rs316009) variant, in linkage disequilibrium (LD) with the variant *SLC22A2* (rs316019), described above, which increased metformin response (Goswami et al. 2016).

In the DPP study a significant interaction of another SNP in *SLC22A2* (rs662301) with metformin has been observed, with the minor allele increasing the diabetes risk in the metformin arm (HR = 1.57, 95% CI 1.09–2.27) (Jablonski et al. 2010).

Recently, Zhou et al. reported a three-stage GWAS from MetGen Consortium (Zhou et al. 2016) of a large multiethnic cohort. Each C-allele copy of *SLC2A2* (rs8192675, T→C) was associated with a 0.17% ($p = 6.6 \times 10^{-14}$) greater HbA1c reduction in response to metformin in 10,577 participants of European ancestry and 0.15% greater HbA1c reduction in 2566 participants of non-European ancestries ($p = 0.005$). The effect was more pronounced in obese patients, as CC-alleles carriers had a 0.33% greater HbA1c reduction compared with TT-allele patients. This may have clinical impact, as it is equivalent of taking 550 mg extra metformin per day. CC-allele carriers may need lower metformin doses, while T-allele carriers may need higher doses to achieve a response. This may be of importance in African Americans where 49% of the population are C-allele homozygotes, compare to 9% in European Americans.

Taken together, *SLC22A2* (rs316019) variant has probably little clinical significance, while the effect of rs662301 and rs8192675 on metformin response should be confirmed.

OCT3 (*SLC22A3*) has a broad distribution, including heart, brain, skeletal muscle, liver, and kidney. There are limited data on the effect of genetic variants of *SLC22A3* on the PK and PD of metformin. The functional analysis of 13 OCT3 polymorphisms (identified in a large multiethnic DNA sample) showed 3 variants with altered metformin specificity (Chen et al. 2010). Thus, metformin uptake was increased by 60% in cells expressing *SLC22A3* (rs68187715 C→T) variant, comparing with those expressing wild type *SLC22A3*, whereas the uptake of metformin was 80% decreased by *SLC22A3* (rs8187725 C→T) and 50% decreased by V423F (1267 G→T) (Chen et al. 2010). However, Tzvetkov et al. (2009) found no statistically significant relationship between 6 common low-activity variants of *SLC22A3* (rs3123634, rs3120137, rs12194182, rs2504927, rs2457576, and rs2292334) and renal clearance of metformin in 103 Caucasian healthy volunteers. Another recent study found that volunteer carriers of *SLC22A3* (rs8187722 and rs2292334) variants had higher metformin concentrations than the wild genotype (Hakooz et al. 2017).

One study found no role of *SLC22A3* (564 G→A, rs3088442) variant on the glycemic response to metformin in 150 patients with newly diagnosed T2D (Ghaffari-Cherati et al. 2016). Although, *SLC22A3* (564 G→A) is a variant in 3′-UTR region of OCT3 which decreases the expression of the transporter.

MATE1 (*SLC47A1*) and **MATE2 (*SLC47A2*)** determine the efflux of metformin into the urine. *SLC47A1* has the best evidence for association of polymorphisms with the glucose-lowering effect of metformin. The intronic variant (rs2289669 G→A) in *SLC47A1* was significantly associated with metformin effect in the Rotterdam study. For each minor A allele (43% of T2D patients) the decrease in HbA1c level was 0.3% ($p = 0.005$), compared with GG genotype, who did not decrease the HbA1c (Becker et al. 2009a). The findings have been replicated in a group of Slovakian patients. The AA carriers of *SLC47A1* rs2289669 (20% of T2D patients) had twofold reduction in HbA1c in response to metformin in comparison with G-allele carriers (1.10 vs. 0.55%, $p = 0.018$) (Tkac et al. 2013). In other group of Caucasian Americans, the metformin effect in AA carriers was greater than in G-allele carriers with a marginal statistical significance ($p = 0.055$) (Choi et al. 2011). In the DPP study, the *SLC47A1* rs8065082 (C→T) variant was associated with lower diabetes incidence in the metformin arm (HR 0.78, 95% CI 0.64–0.96, p 0.02), while major allele homozygotes CC (30% of the European population) did not benefit from metformin. Moreover, the rs8065082 variant was in tight LD with rs2289669, and the effect was consistent with the one described in the above studies (Jablonski et al. 2010).

The *SLC47A1* reduced-expression promoter variant (rs2252281, T→C) have been associated with enhanced metformin response in 57 healthy volunteers and 145 diabetic patients (Stocker et al. 2013). Diabetic patients, homozygous for the *SLC47A1* variant allele, without reduced-function variants of OCT1, had a 15% greater relative reduction in Hb1Ac compared to patients carrying the *SLC47A1* wild type allele. It is possible that the variant allele of OCT1 would result in lower drug levels and would mask the effects of the MATE1 variant. The magnitude of the effect is important, as a typical T2D patient with a baseline HbA1c of 8% receiving metformin monotherapy would have their HbA1c decreased by an additional 1.2% if they were a carrier of the MATE1 variant allele instead of wild type MATE1 (Stocker et al. 2013).

Two isoforms of MATE2 have been identified, one of them, MATE2-K, is expressed specifically in the kidney. The MATE2-K gain-of-function 5′-UTR variant of (rs12943590, G→A) was significantly associated with enhanced promoter activity and an attenuated response to metformin in newly diagnosed US diabetic patients (Choi et al. 2011). The variant carriers may eliminate metformin more quickly, with lower therapeutic effect (only a 2.7% relative reduction in HbA1c in homozygous AA vs. 15% in all others). The minor allele frequency was 22% in African Americans and 28% in Caucasians (Choi et al. 2011).

The MATE2 enhanced-expression promoter variant (rs12943590, G→A) was associated with increased renal and secretory clearance of metformin (22% and 26%, respectively) and reduced response to metformin in healthy volunteers (Stocker et al. 2013). The MATE1 and MATE2 genotypes were the only significant predictors of metformin response (and not the sex, age, BMI, fasting glucose, fasting insulin). Each genotype explained 7% of the variability in the glucose-lowering effect of metformin (Stocker et al. 2013).

Finally, despite all associations mentioned above, each individual SNP in transporters accounts for only a small fraction of the response to metformin among T2D patients. One possible explanation is that metformin disposition is controlled by multiple transporters. The clinical utility of these transporter gene polymorphisms remains to be demonstrated.

Ataxia-telangiectasia mutated gene (*ATM*) Zhou et al. described for the first time how GWAS could be applied to the glycemic response to metformin (Zhou et al. 2011). In the Scottish participants from GoDARTS and UK patients from UKPDS a genome-wide association 'signal' on chromosome 11 has been reported in a locus containing seven genes. The minor C-allele of the most strongly associated SNP, rs11212617 increased the ability of metformin to achieve HbA1c below 7% with OR of 1.35 for each minor C-allele (allele frequency of 44%) and OR of 3.3 for two alleles (19% of the patients), compared with those without any variant. The authors suggested that the most likely candidate is the ATM gene, and the researchers have shown *in vitro* that ATM is involved in AMPK's activation by metformin. The data have been replicated in a meta-analysis (van Leeuwen et al. 2012) and in a study on Chinese diabetic patients (Zhou et al. 2014). However, the association between rs11212617 variant and the incidence of diabetes might be confined to populations of European descent, as no association was observed in people of American ancestry, enrolled in DPP study (Florez et al. 2012). Moreover, it is a matter of debate if the ATM gene is the causal gene at this region, as the ATM inhibitor used in the *in vitro* studies, it has since been shown to inhibit OCT1 transport and prevent metformin uptake into hepatic cells (Woods et al. 2012).

AMPK subunits, AMPK activator *(STK11)* and other associations There were 91 SNPs nominally associated with the metformin intervention in the DPP

study. Among most significantly associated variants with metformin treatment were in genes encoding AMPK subunits (*PRKAA1, PRKAA2, PRKAB2*), AMPK activator (*STK11*), mitochondrial complex I inhibitor calpain 10 (*CAPN10*), sulfonylurea receptor 1 and Kir6.2 subunits of the ATP-sensitive K(+) channel (*ABCC8-KCNJ11*) and metformin transporters (*SLC47A1, SLC22A1*) (Jablonski et al. 2010).

In a more recent study, carriers of minor G-allele of *CAPN10* (rs3792269) (24% in European Caucasian T2D patients) had a smaller probability of achieving HbA1c level <7% with an OR of 0.27 per variant allele (OR = 0.27, 95% CI 0.12–0.62, $p = 0.002$) and a smaller reduction in HbA1c during the first 6 months of metformin treatment (Tkáč et al. 2015).

It has been proposed that genetic polymorphisms in transcription factors (TF) that regulate several metformin transporters may have greater effects on metformin PK and PD than variants in a single transporter. In a study involving 440 T2D patients, 40 SNPs in 5 TF were associated with a change in HbA1c levels after metformin initiation. Among the 40 SNPs, six variants in two genes were associated also with metformin clearance. Five SNPs were in the specificity protein 1 (SP1) and one was in the activating enhancer-binding protein (AP)2, TF that controls the expression of metformin transporters. The SP1 (rs784892) variant (allele frequency of ~11%) was the most strongly associated with both metformin PK and PD, with β coefficients of −76.9 ml/min per G allele ($p = 0.02$) and −0.32 HbA1c per G allele ($p = 0.008$), respectively. Other 23 genetic polymorphisms in PPAR-α and HNF4-a were associated with metformin response, confirming the findings from DPP (Jablonski et al. 2010), but none of which were explained by a PK mechanism. PPAR-α and HNF4-α are multifunctional TF and their gene expression levels were strongly correlated with expression of metformin transporters (Goswami et al. 2014).

A recent combined non-linear mixed effect model with computational genetic techniques identified predictors of metformin response in 1056 T2D patients (Goswami et al. 2016). Nine variants in 8 genes accounted for about one-third of the variability in the progression of HbA1c levels on metformin. Of the 9 variants, *CSMD1* (rs2954625), *VPS13C* (rs12907856), and *SLC22A2 (*rs3160009) had greater effects on HbA1c levels than the clinical parameters, accounting for about 5%, 6%, and 8% of the HbA1c variability. The model predicted that the HbA1c levels start to increase in patients on metformin at about 321 days, at a rate of 0.1% per year, for the next 3 years; while in patients not treated with metformin, HbA1c levels increase at a steady-state rate of about 0.16% per year (Goswami et al. 2016). Two SNPs (rs2617102, rs2954625) in the *CSMD1* gene (CUB and Sushi multiple domains 1) had the worst impact on disease progression, although the mechanism is unknown. Polymorphisms in genes *SLC22A2, FOXN3, EMILIN2*, and *WWOX* were associated with lower HbA1c progression.

Insulin secretagogues (sulfonylureas and meglitinides)

After metformin, sulfonylureas (SUs) are the most commonly used oral antidiabetic agents for the treatment of T2D. SUs are insulin secretagogues by inducing closure of the ATP-sensitive potassium channel (K_{ATP}) in pancreatic β cells. The K_{ATP} is constituted by 4 pore-forming Kir6.2 subunits encoded by *KNJ11* gene. The outer part of the channel is composed of 4 SUs receptor 1 (SUR1) subunits encoded by ATP-binding cassette transporter, subfamily C, member 8 (*ABCC8*) gene. The binding of SUs to SUR1 led to the closure of the K_{ATP}, increased concentrations of intracellular potassium, depolarization of the β cells membrane, Ca^{2+} influx and insulin secretion.

Although most of the T2D patients responded well to SUs therapy, there is a significant rate of SUs monotherapy failure that reaches 20% at 1 year (Wright et al. 2002) and 34% at 5 years (Kahn et al. 2006). Earlier studies showed that higher baseline FPG and HbA1c levels are associated with a greater response to oral therapy (including SUs). A more recent analysis showed that only baseline HbA1c and shorter diabetes duration were significantly associated with SUs response; while race, smoking, lipid levels, blood pressure, kidney function, and comorbidities did not predict treatment response (Martono et al. 2015).

With the progress of GWAS, over 100 susceptibility loci for T2D have been identified. Many of them have an impact on insulin secretion and/or sensitivity. Consequently, they might influence the efficacy of insulin secretagogue and/or sensitizer. A recent study examined the combined effect of T2D-related loci on the efficacy of two oral drugs: repaglinide (insulin secretagogue) and rosiglitazone (insulin sensitizer) in newly diagnosed T2D patients. The reductions in FPG and HbA1c levels after one year of treatment with repaglinide or rosiglitazone were associated with the genetic score of 22 SNPs, respectively 23 SNPs, after adjusting for gender, age and BMI (Chen et al. 2016). Similarly, Ren et al. described a model composed of clinical and genetic markers, which predicts SUs response, with an area under the receiving operator characteristic curve

(AUC) of 0.77 (Ren et al. 2016). They identified 4 genes (*FMO2, UGT2B15, FMO3,* and *CYP51A1*) related to drug metabolism, 2 transporter genes (*ABCB11*and *ABCC5*) and one kinase (*MAPK1*) that have been associated with SUs response. Several other polymorphisms have been associated in pharmacogenetic studies with SUs response (Table 2).

SUs are inactivated primarily by the cytochrome P450 2C9. According to GoDARTS study, about 6% of the population carry two reduced-function variants in the ***CYP2C9* gene** (rs1799853 C→T, rs1057910 A→C) associated with greater response to SUs (OR 3.44 (1.65, 7.15), $p = 0.0009$) due to increased drug concentration (Zhou et al. 2010). These patients would need lower starting doses of SUs to avoid hypoglycemia. The minor alleles frequencies of rs1799853 and rs1057910 were 13.4% and 7.3%, respectively.

In another study on 156 Caucasian patients, no influence of genotype on HbA1c reduction in response to SUs was found, neither for *CYP2C9* nor for other polymorphisms discussed below (ABCC8 rs757110, KCNJ11 rs5215, KCNJ11 rs5219). The data should be interpreted carefully because the patients included in the study were already treated with SUs for more than 10 years, so patients who did not achieve an adequate glycemic control or who had hypoglycemia may already be changed to other therapies. Therefore, the authors did not conclude, that there is no effect of CYP2C9, ABCC8, or KCNJ11 genotype on SUs response (Klen et al. 2014). However, in elderly patients, CYP2C9 variants significantly increased the risk of hypoglycemia. Carriers of one or two variant alleles had 0.79 ± 1.7 or 2.67 ± 4.6 hypoglycemic events, while homozygous for reference allele suffered 0.36 ± 0.98 events ($p = 0.014$) (Klen et al. 2014).

Results similar to that of GoDARTS' findings have been reported in the Asian population (Surendiran et al. 2011; Zeng et al. 2016). A smaller study in Indian patients has shown a significant association ($p < 0.001$) between *CYP2C9* variants (rs1799853 and rs1057910) and controlled diabetic status to therapy with glibenclamide, while the reference genotype of *CYP2C9* was associated with uncontrolled diabetes (Surendiran et al. 2011). A larger prospective cohort study on Chinese patients confirm the effect of rs1057910 (A→C) polymorphism on gliclazide response. Carriers of C–risk allele (AC and CC, around 10% of population) had greater reduction of FPG (3.6 vs 3.0 mmol/L, $p < 0.001$) and a higher rate of achieving HbA1c < 7.0 mmol/L (51.4 vs 32.3%, $p < 0.001$) compared with AA genotypes; whereas the differences in hypoglycemic events were not statistically significant (Zeng et al. 2016).

TCF7L2 encodes a TF expressed in pancreatic β cells, that it is a master regulator of insulin production and processing. Two *TCF7L2* polymorphisms, rs12255372(G→T) and rs7903146(C→T) have been found to substantially contribute to the risk of T2D.

In a cohort of patients from the GoDARTS study, Pearson et al. revealed that TT risk-allele homozygotes of *TCF7L2,* rs12255372 (∼12% of the population) had a lower response rate to SUs with an OR for the failure of 1.95 (95% CI 1.23–3.06; P 0.005), comparing GG genotype. A slightly weaker association was seen in TT risk-allele homozygotes of *TCF7L2,* rs7903146 (∼12.5% of the population) (OR 1.73) (Pearson et al. 2007). The per-allele OR for treatment failure was intermediate of 1.28 for rs12255372 (*p* 0.014) and 1.27 for rs7903146 (*p* 0.017). The risk allele frequency in the population was 58% for each variant (Pearson et al. 2007).

Two other European studies strengthen GoDARTS' findings regarding the influence of *TCF7L2* (rs7903146) polymorphism on SUs response. Thus, in German T2D patients the T-allele carriers had OR SUs failure of 1.57 (CI, 95% (1.01–2.45); $p = 0.046$) (Holstein et al. 2011), while in Slovakian population, variant T-allele carriers had lower HbA1c reductions compared with reference homozygotes (0.86% vs. 1.16%, $p = 0.003$) (Schroner, Javorsky, et al. 2011). The above three studies had a quite similar design: use of metformin plus SUs, assessment of outcome after 6 months of SUs and use of similar outcome measures (SUs failure defined by HbA1c >7%). They strongly suggest that Caucasian patients with rs7903146 polymorphism have decreased efficacy of SUs.

A more recent study (Dhawan and Padh 2016) confirmed the effect of *TCF7L2* (rs12255372) variant on SUs response in Indian T2D population. Thus, 70.5% of T-risk allele carriers failed to achieve HbA1c below 6.5%, compared to 51.6% in GG homozygotes. The association of *TCF7L2* (rs7903146) variant with SUs response did not reach statistical significance, probably due to smaller size study (250 T2D patients). Dhawan et al identified another *TCF7L2* (rs4506565 A→T) variant that influenced response to SUs, with more treatment failure in the TT homozygotes (Dhawan and Padh 2016).

A recent report (Srinivasan et al. 2018) from The Study to Understand the Genetics of the Acute Response to Metformin and Glipizide in Humans (SUGAR-MGH) showed that TCF7L2 (rs7903146, C→T) influences acute responses to glipizide and metformin. High-risk TT-carriers achieved a trough glucose level faster than CC-carriers after a single dose of glipizide and had a steeper slope to glucose trough. However, this is in the opposite direction to GoDARTS' findings.

Few studies have reported the association of ***KCNQ1*** variants with a response to SUs in T2D. The *KCNQ1* gene encodes the pore-forming subunit of a voltage-gated K + channel (KVLQT1) with a role in cardiac repolarization and probably insulin secretion in pancreatic β-cells. The *KCNQ1* gene has been identified as a susceptibility locus for T2D.

A study in T2D Slovakian patients treated with SUs plus metformin for 6 months found a significant genotype-specific effect of *KCNQ1* (rs163184, T→G) on the reduction in FPG, with a lower reduction in GG homozygous patients (32% din population) compared with T-carriers (1.04 ± 0.18 mmol/L vs. 1.58 ± 0.13 mmol/L, $p = 0.016$) (Schroner, Dobrikova et al. 2011).

A recent study in T2D Chinese patients, carriers of one of the two *KCNQ1* polymorphisms *(*rs2237892, C →T and rs2237895 A→C) had improved the efficiency of gliclazide (Li et al. 2017). The rs2237892 T and rs2237895 C variant alleles were associated with treatment success (decrease in HbA1c) compared with the C reference allele (OR = 2.533, 95% CI: 1.283–4.999, $p = 0.007$) and A reference allele (OR = 2.360, 95% CI: 1.225–4.550, $p = 0.009$), respectively. Unfortunately, two additional large studies in Chinese population could not confirm the de effect of *KCNQ1* polymorphism, rs2237892 on SUs response (Ren et al. 2014; Duan et al. 2016).

Duan et al. investigated the association of two *KCNQ1* polymorphisms (rs2237892, C →T and rs2237897, C →T) with the efficacy of gliclazide in a large cohort of Chinese newly diagnosed T2D patients (Duan et al. 2016). Carriers of the rs2237897T variant allele achieved greater reduction in FPG (3.9 ± 2.6 vs. 3.2 ± 2.4 mmol/l, $p = 0.003$) and a higher rate of treatment success (61.1 vs. 44.5%, $p < 0.001$ for attending FPG less than 7.0 mmol/l), compared with CC genotype group. No significant effect of rs2237892 variant on SUs response has been noted (Duan et al. 2016).

Another study, including 747 Chinese T2D patients, was carried out to test the association of 44 SNPs in 27 genes with the efficacy of glibenclamide (Ren et al. 2014). No association between two *KCNQ1* polymorphisms (rs2237892 and rs12720449) and glibenclamide response was found. Similarly, another recent study, including 209 T2D Indian patients, found no association between *KCNQ1* or *TCF7L2* polymorphisms with glibenclamide treatment response (Phani et al. 2017).

KCNJ11 gene encodes Kir6.2 subunit of the K_{ATP}. *KCNJ11* (E23K, rs5219) polymorphism causes substitution of glutamate (E) by lysine (K) in Kir6.2 and was associated with T2D in genetic association studies. Elegant studies in neonatal diabetes have shown that even mutated KATP channels (*KCNJ11*) could be closed by SU, allowing switching from insulin therapy to SU in severe hyperglycemic, insulin deficient young patients (Pearson et al. 2006, Ioacara et al. 2017).

Pharmacogenetic studies in T2D that tested the association of *KCNJ11* polymorphism with the glucose-lowering effect of SUs generated controversial results.

Studies in Caucasian populations showed either no association (Nikolac et al. 2009; Klen et al. 2014), a better SUs response in *KCNJ11* K-allele (Javorsky et al. 2012) or a higher rate of secondary SUs failure in *KCNJ11* K-allele carriers (Sesti et al. 2006). Thus, in a large Italian cohort of T2D patients, the carriers of *KCNJ11* (rs5219) variant had a relative risk for secondary failure of 1.69 (95% CI: 1.02–2.78; *p* 0.04), as compared with non-carriers and after adjusting for age, gender, FPG, HbA1c, duration of diabetes and age at diagnosis (Sesti et al. 2006). The K-allele frequency was 51.4% (heterozygous) and 10.1% (homozygous). In contrast, a study in Slovakian patients showed that *KCNJ11* K-allele carriers had a greater decrease in HbA1c compared with wild-type EE by 0.16% (95% CI 0.01–0.32, $p = 0.038$) per each K-allele (Javorsky et al. 2012). However, *KCNJ11* rs5219 and rs5215 variants were not associated with increased risk of hypoglycemia in the Slovakian population (Klen et al. 2014). The duration of SUs exposure of the patients was different in these two investigations making comparison and potential clinical inference difficult: the Italian cohort patients were treated with maximal dose of glibenclamid for an average of 12 years (Sesti et al. 2006), while Slovakian patients with SUs duration of 6 months (Javorsky et al. 2012).

There are two other studies on Caucasians which did not show a significant effect of E23K polymorphism on SUs response (Nikolac et al. 2009; Klen et al. 2014). They should be interpreted cautiously because they were cross-sectional studies and the patients included were treated with SU for many years, so patients who did not achieve an adequate glycemic control may already be switched to other therapies.

In Asian population, a prospective study showed that *KCNJ11* (rs5219) variant was a predictor for glibenclamide treatment failure (Phani et al. 2017), while another common variant of *KCNJ11* (rs5210), was associated with a better gliclazide response in a large cohort of Chinese T2D patients (Feng et al. 2008).

Poor SUs response in E23K variant carriers have been reported by two other studies, one on Egyptians T2D and another on Japanese. El-sisi et al. reported an increase in the relative risk of SUs failure (OR = 1.65;

95% CI: 1.04–2.60; $p = 0.04$) defined by HbA1c >8% on SUs therapy, as indicator of SUs failure (El-Sisi et al. 2011). In a Japanese study, the glycemic control of K-variant carriers deteriorated faster and required insulin therapy earlier (7.7 ± 4.6 years) compared with wild-type homozygotes (11.1 ± 6.1 years) (Shimajiri et al. 2013). Although rs5219 is strongly associated with T2D risk, there is weak evidence that the variant increases the risk of SUs' failure.

***ABCC8* gene** encoding SUR1 subunit of the K_{ATP} channel, have been associated with T2D risk. In Chinese patients, *ABCC8* (Ser1369Ala, rs757110, T→G) polymorphism was associated with enhanced response to gliclazide therapy in risk allele carriers. The decrease in HbA1c was more important in G carriers than in TT genotype (TG + GG, 1.6% vs. TT, 0.76%; $p = 0.044$) (Zhang et al. 2007). The allele frequencies were 33% TT, 47% TG and 20% GG, respectively. This implies that G allele carriers are hypersensitivity to SUs, while TT genotype will need more effective therapeutic agents. However, this polymorphism has not been associated with the risk of severe hypoglycemia in SUs treated patients (Sato et al. 2010; Klen et al. 2014).

In another Chinese cohort, *ABCC8* (rs757110) variant was significantly associated with an increased response to gliclazide therapy. Carriers of G-risk allele had OR of 1.4 in heterozygous (95% CI 1.0–2.1, p 0.06) and 2.2 in homozygous genotype (1.4 –3.6, p 0.001) for a response to gliclazide therapy compared with wild-type homozygote (TT)(Feng et al. 2008).

In a cohort of T2D Caucasian patients, two other *ABCC8* gene variants have been associated with SUs' response (Nikolac et al. 2009). Homozygous TT variant carriers of the *ABCC8* (rs1799854, C→T) had higher HbA1c compared to heterozygotes and wild-type CC genotypes (TT: 8.1% vs. CT: 7.6% vs. CC: 6.9%; $p = 0.009$). Opposite results were reported for the *ABCC8* (rs1799859). The risk AA-allele carriers had significantly lower HbA1c levels compared with heterozygotes and wild-type homozygotes (AA: 6.3% vs. GA: 7.1% vs. GG: 7.8%; $p < 0.0001$)] (Nikolac et al. 2009).

A common variant in ***IRS-1*** gene (Gly972Arg, rs1801278), associated with T2D risk, has been shown to increase twice the risk for secondary failure to SUs in a large Caucasian cohort of T2D patients (OR 2.0 (1.38–3.86), $p = 0.038$) (Sesti et al. 2004). The genotype frequency of the Arg (972) IRS-1 variant was 11.3%.

A quite similar study in Egyptian patients confirmed that *IRS-1* gene (rs1801278) variant conferred added risk of SUs failure (OR 1.75; 95% CI: 1.08–12.4; $p = 0.041$) (El-Sisi et al. 2011). The variant allele frequency was 14%.

However, in the Chinese population, the frequency of this variant was very low (0.9%) and didn't influence SUs response (Feng et al. 2008).

Incretin therapies

The incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are gut hormones secreted from enteroendocrine cells into the blood rapidly and transiently following food ingestion. Both incretins are rapidly degraded by an enzyme called dipeptidyl peptidase 4 (DPP4).

Two incretin-based therapeutic agents have been developed recently: GLP-1 receptor agonists (GLP-1RA) and DPP-4 inhibitors (DPP-4i). However, a significant proportion of patients do not respond to incretin-based agents and there are no specific predictors of treatment response.

A meta-analysis of 98 randomized controlled trials (RCTs) of DPP-4i (sitagliptin, vildagliptin, linagliptin, saxagliptin, and alogliptin) showed that baseline HbA1c and FPG levels are the only predictors of HbA1c response to DPP-4i in T2D patients. Other factors like age, previous oral treatment, duration of therapy had no predictive role (Esposito et al. 2015). Another retrospective analysis of three phase-3 studies confirmed that baseline FPG and HbA1c predicted the response to linagliptin (Del Prato et al. 2016). Additionally, linagliptin was more effective in normal body weight patients without metabolic syndrome (MetS) (Del Prato et al. 2016).

There is substantial variability in the glucose-lowering effect of DPP-4i in clinical practice. A large retrospective study, using a UK primary care database, assessed the variability and determinants of glycemic response to DPP-4i (Mamza et al. 2016). Overall, the study shows a 0.5% reduction in HbA1c at 1 year after DPP-4i was added. Independent predictors of achieving optimal glycemic control (HbA1c <7%) were: the use of metformin (OR = 2.58) and use of metformin plus SUs (OR = 1.42) as opposed to no use. Conversely, a higher baseline HbA1c and longer diabetes duration were associated with a lower probability of achieving an HbA1c <7%.

The glucose-lowering efficacy of DPP-4i was reported to be greater in Asians than in non-Asians (Kim et al. 2013). In the Japanese population, the factors independently associated with the glucose-lowering effect of sitagliptin at 24 months were: the baseline and at 3 months HbA1c levels, the decrease in body weight and diabetes duration (Nishimura et al. 2015). The factors that were found to contribute to the secondary

ineffectiveness of sitagliptin, among the patients who showed improvement of HbA1c at 3 months, were body weight gain (between 3 and 24 months) and longer duration of diabetes (Nishimura et al. 2015).

Dennis et al have recently reported that higher body mass index and increased markers of insulin resistance, such as triglycerides, fasting C peptide, and HOMA2-IR were associated with reduced HbA1c response to DPP-4i (at 6 months) and less durable response (after 3 years). Moreover, the drug response was independent of sex, age, ethnicity, diabetes duration, baseline HbA1c, and renal function (Dennis et al. 2018).

Finally, hemoglobin glycation index (HGI) has been suggested as a predictor of therapeutic responses to DPP-4i in T2D patients (Chen YW et al. 2017). The HGI has been calculated as the difference between observed HbA1c and estimated HbA1c from a linear regression equation including observed fasting plasma glucose. The patients with a high HGI had a significantly greater improvement in HbA1c compared to those with a low HGI (−1.9 versus −0.3%).

GLP- 1RA stimulate insulin secretion and inhibit glucagon secretion as well as decrease glucose absorption by slowing gastric emptying. Current guidelines recommend GLP-1RA as add-on therapy to metformin or after dual therapy failure (DC 2018; ADA 2019), while others recommend GLP-1RA only as third-line therapy (NICE 2017). Three molecules of the class (lixisenatide, exenatide, and liraglutide) are approved by regulatory authorities for use in combination with insulin. There is a significant disparity in GLP-1RA response, from large effect to lack of response in several patients.

A recent meta-analysis identified few predictor factors of GLP-1RA response (Monami et al. 2017). Long-acting molecules are more effective in patients with fasting hyperglycemia, while short-acting agents are more efficient in patients with postprandial hyperglycemia. Duration of diabetes and obesity do not influence the glucose-lowering effect of GLP-1 RA. Liraglutide seems to be less successful in older non-Caucasian patients.

In routine clinical practice, higher baseline HbA1c was the only independent predictor of glycemic response to exenatide once-weekly, while previous DDP-4i and higher BMI were predictors of significant weight loss (Gorgojo-Martinez et al. 2018). Similar real-world data has been reported for liraglutide response. Thus, baseline HbA1c was the strongest predictor of glycemic response to liraglutide after a period of 6 months (Berkovic et al. 2017) or 12 months (Simioni et al. 2018), followed by prior insulin therapy, diabetes duration, and female gender. Baseline BMI, HbA1c, systolic BP, and cholesterol levels were independent predictors of maintaining the glycemic response for up to 36 months of therapy (Berkovic et al. 2017).

In Japanese patients, the response to GLP-1 RA therapy (exenatide or liraglutide) was associated with the BMI, the preprandial glucose levels during the 2 days after the initiation of GLP-1 RA, and the treatment used before GLP-1 RA (Imai et al. 2014).

Several diabetes risk-related gene polymorphisms have been recently involved in incretin response (Table 3).

A variant of ***CTRB1/2*** (rs7202877 T→G) gene, encoding chymotrypsinogen 1 and 2, has been previously shown to be protective against diabetes development. Carriers of the minor G allele (10% of the patients) showed a 0.5% smaller reduction in HbA1c after gliptin therapy (84% with sitagliptin) compared with TT-allele patients ('t Hart et al. 2013).

Two variants of diabetes risk-related gene ***TCF7L2*** (rs7903146, rs12255372) have been shown to impair GLP-1-induced insulin secretion (Schafer et al. 2007). One of them (rs7903146 C→T) was associated with a smaller reduction in HbA1c after linagliptin therapy in TT-allele carriers by 0.25% compared with CC-allele patients (Zimdahl et al. 2014).

GLP-1R polymorphisms have been associated with both DPP-4i and GLP-1RA therapeutic responses. Thus, genetic variations of *GLP-1R* (rs3765467 G→A) have been associated with the glucose-lowering effect of DPP-4i in Korean patients (Han et al. 2016). Compared to the major GG-genotype, significantly more A-risk allele carriers (57% of the population) showed a greater HbA1c reduction (1.3 ± 1.1 vs $0.9 \pm 1.2\%$; $p = 0.022$). Vildagliptin and sitagliptin were the most used DPP-4i. Recently, Suzuki et al. reported that this GLP-1R polymorphism is more common in Japanese population compared to the European population (Suzuki et al. 2019) and it may explain the better response to incretin drugs, observed in the Asian population (Kim et al. 2013). Another *GLP-1R* variant (rs6923761G →A) was associated with a reduced response to gliptins (sitagliptin or vildagliptin) (Javorsky et al. 2016). Risk alleles-AA carriers (13% of patients) have a smaller HbA1c reduction compared with reference allele carriers (0.12% vs. 0.8%; $p = 0.008$). The same *GLP-1R* variant (rs6923761) has been studied in relation to the glucose-lowering effect of liraglutide. Although not statistically significant, it was a trend to a greater decrease of HbA1c in A allele carriers compared to wild type GG ($0.87 \pm 1.2\%$ vs. $1.2 \pm 1.1\%$; ns) (de Luis et al. 2015).

Lin et al. identified two *GLP-1R* gene polymorphisms, with more than 20% allele frequency, in 36 patients with T2D (rs3765467 C→T, rs761386 C → T), that showed significant associations in opposite direction with changes in the standard deviation of plasma

glucose upon exenatide treatment (GLP-1RA)($p = 0.041$ for rs3765467; $p = 0.019$ for rs761386) (Lin et al. 2015). However, the association became statistically insignificant after multiple linear regression analyses.

Two common polymorphisms in diabetes risk-related gene ***CDKAL1*** (rs7754840 G→C, rs7756992 A→G) have been associated with greater DPP-4i response, in 512 T2D patients. Unlike other diabetes drugs, HbA1c reduction was significantly greater in patients taking DPP-4i, who carried rs7754840 (0.4% in GG; 0.5% in GC; 0.8% in CC; $p = 0.02$) and rs7756992 (0.4% in AA; 0.5% in AG; 0.8% in GG; $p = 0.01$). The HbA1c reduction remained significantly associated with *CDKAL1* genotype for more than 12 months, even after adjusting for age, sex, BMI, baseline HbA1c, duration of diabetes, and concomitant anti-diabetic drugs (Osada et al. 2016). The minor allele frequencies of rs7754840 and rs7756992 were 43% and 48.1%.

In a large cohort of Malaysian T2D patients treated with DPP-4i (sitagliptin, linagliptin or vildagliptin), patients with normal triglyceride levels, less than 1.7 mmol/l (OR: 2.2.; 95% CI: 1.031–4.723), diastolic BP less than 90 mmHg (OR: 1.7; 95% CI: 1.009–2.892) and ***KCNJ11*** rs2285676 (genotype CC) were more likely to achieve a HbA1c less than or equal to 7.0% on DPP-4i therapy (OR: 2.1; 95% CI: 1.094–3.923)(Jamaluddin et al. 2016). The age and duration of T2D had no significant influence on glycemic response to DPP-4i. The frequency of *KCNJ11* rs2285676 (C→T) was: 30.8% (CC), 48.6% (CT) and 20.5% (TT).

In Caucasian T2D patients, ***KCNQ1*** (rs163184T→G) variant was associated with the glucose-lowering effect of DPP-4i. If replicated, this finding might have a clinical impact as the difference in HbA1c was 0.6% between the TT and GG carriers (Gotthardova et al. 2017).

Using a GWAS-based approach in a two-stage study, Liao et al. identified that ***PRKD1*** rs57803087 polymorphism was strongly associated with DPP-4i therapeutic efficacy ($p = 3.2 \times 10^{-6}$). *PRKD1* is a pancreatic β-cell protein kinase associated with many β-cell functions (Liao et al. 2017).

Recently, two polymorphisms in ***DPP4*** (rs2909451 C→T; rs759717 G→C) were associated with increased DPP-4 activity during sitagliptin treatment in 27 T2D patients with hypertension and 38 healthy controls (Wilson et al. 2017).

Sodium-glucose co-transporter 2 (SGLT2) inhibitors

SGLT2 is responsible for most glucose reabsorption in the kidney and represents the target of a novel class of antidiabetic drugs. SGLT2 inhibitors (SGLT2i) reduce hyperglycemia by increasing urinary glucose excretion. Additionally, they reduce body weight and blood pressure. An ample analysis of the dapagliflozin phase 3 studies have shown that are no predictors of response to SGLT2i therapy (Bujac et al. 2014).

SGLT2 is encoded by the *SLC5A2* gene. It has been shown that many, rare inactivating mutations in *SLC5A2* cause familial renal glucosuria and protect against hyperglycemia.

Early data, by Enigk et al. suggest that common *SLC5A2* variants could explain some of the variation in body weight and glucose metabolism in nondiabetic subjects. They showed that the rs9934336 genotype was associated with lower glucose concentrations during OGTT (Enigk et al. 2011). However, in a recent cross-sectional study of 2229 individuals at increased risk for T2D, none of the common *SLC5A2* polymorphisms (rs9924771G→A, rs9934336G→A, rs3813008G→C, rs3116150G→A) significantly influenced plasma glucose or HbA1c. Furthermore, no interaction was observed between the genotypes and the change from baseline HbA1c after 24 weeks of treatment with the SGLT2i empagliflozin, in 603 T2D patients enrolled in four phase-3 trials (Zimdahl et al. 2017).

SGLT2 inhibitors are metabolized by several UGT isozymes. The UGTs polymorphisms are expected to influence the PK and PD of metabolizing drugs. Francke et al. have shown that UGT1A9*3 allele carriers ($n = 3$) showed 54% higher dose-normalized steady-state AUC of canagliflozin compared with wildtype UGT1A9*1/*1 subjects ($n = 62$) (10,613.3 ng·h/mL vs. 6895.0 ng·h/mL) (Francke et al. 2015). Data has been confirmed in a population PK analysis of 1616 healthy volunteers and T2D patients (Hoeben et al. 2015). UGT1A9*3 allele carriers ($n = 21$) showed higher median dose-normalized AUC values compared with subjects not carrying this allele ($n = 700$) (ratio = 1.26; 95% CI = 1.08–1.44). However, the magnitude of the effect was small and, therefore, not considered clinically relevant for canagliflozin dosage adjustment.

The variety of diabetic drug pharmacogenomics studies preclude a systematic analysis of the current data, a required step toward treatment personalization. There are many differences in study designs, associated medications, clinical endpoints, regimens and dosage used, sample sizes, ethnic variability, etc. The definition of clinical endpoints varies greatly, where some have assessed the HbA1c response, others used blood glucose levels; the response was evaluated within weeks or months (primary failure) or after long-term therapy (secondary failure). Some authors did not use the maximum dose

compared with others which did and this may impact the drug failure. The sample size of the studies varies largely from a hundred to thousands. Most studies only represent an ethnic group or have not been replicated in different groups, therefore, generalizing the results is difficult. Additional multi-centered, multi-ethnic, larger prospective studies with longer duration and well-defined endpoints would provide stronger evidence which may allow genetic testing to predict clinical outcomes of diabetes drug in the future.

Additional multi-omics predictive data of diabetes drug's response

The gut microbiome affects drug metabolism and bioavailability and it is associated with disease risk and drug exposure. There is evidence showing that glucose-lowering drugs alter gut microbiota, but also intestinal microbiota exert a positive effect on antidiabetic drugs. Thus, there is a specific signature of gut microbiota in metformin users (Forslund et al. 2015). Metformin induces alteration of gut microbiota, which explains part of the metformin intolerance and efficacy. Recently, it has been confirmed that metformin has a strong effect on the gut microbiome (Wu et al. 2017), while, the use of a microbiome modulator improves metformin intolerance and glycemic control (Burton et al. 2015).

α-glucosidase inhibitors (α-GI) such as acarbose, voglibose, and miglitol reversibly bind to human α-glucosidases, inhibiting carbohydrate hydrolysis and glucose absorption in the upper small intestine, and consequently reduce postprandial hyperglycemia. Recently, it has been shown that there is a high similarity between human and bacterial α-glucosidases and the gut bacterial α-glucosidases can process dietary carbohydrates as well as be effectively inhibited by the α-GI (Tan et al. 2018). The differential response to Acarbose treatment in patients with T2D has been related to baseline gut microbiota. Thus, Bacteroides-enriched microbiota predicted a greater response to acarbose therapy compared to Prevotella abundance (Gu et al. 2017).

Incretin-based therapies are efficient in controlling T2D hyperglycemia, though some individuals develop GLP-1 resistance. It has been demonstrated recently, that GLP-1 sensitivity is modulated by gut bacteria through NO signaling in the enteric nervous system. A specific gut microbiota architecture impairs the action GLP-1 on gut-brain-regulated insulin secretion and gastric emptying (Grasset et al. 2017). Mounting evidence shows that the release of incretin hormones from enteroendocrine cells can be influenced by the presence of bacteria and their metabolites within the gut. Thus, bacterial metabolites like secondary bile acids, short-chain fatty acids (SCFAs) and lipopolysaccharide (LPS) are potent GLP-1 secretagogues (Martin et al. 2019). On the other hand, microbiota possess DPP-4-like activity, which may be reduced by the DPP-4 inhibitor, vildagliptin (Martin et al. 2019).

Metabolomics is an alternative approach for predicting response to treatment. A recent study identified several metabolites that were associated with the diabetes drugs response to metformin and/or SUs (den Ouden et al. 2016). In patients treated with metformin, high levels of liver metabolites 3-hydroxybutanoic acid and 2-hydroxybutanoic acid at diagnosis predicted a greater metformin response. In patients treated with SUs, high levels of fumaric acid were associated with a larger decrease in HbA1c after 5 years. Similar results have been reported by different researchers (Rottroff et al. 2016), who showed that metformin-mediated glucose response in non-diabetic individuals was significantly correlated with metabolites like, 2-hydroxybutanoic acid, 2-deoxytetronic acid, fumaric acid, glycine, and malic acid. More recently (Park et al. 2018), different metabolite profiles have been identified in diabetic patients treated with metformin for 6 months. Particularly, citric acid, myoinositol, and hippuric acid levels showed significant differences between metformin responders and non-responders.

The serum metabolic profiling of gliclazide-modified-release-treated T2D patients has showed that a combined metabolites panel, including HbA1c, methyl hexadecanoate, methyl 15,8,11,14-eicosatrienoate and 5,8,11,14,17-eicosapentaenoic acid predicted very well gliclazide response (Zhou et al. 2018).

These results need to be replicated and new metabolic biomarkers for antidiabetic drugs response are expected to enhance personalized treatment in T2D.

Epigenetic modifications alter gene expression without sequence variations in many chronic diseases, including diabetes (Fodor et al. 2015). Different genes responsible for the variability in diabetes drug response are subjected to epigenetic regulation such as histone-acetylation or DNA-methylation and miRNAs, making them responsive to environmental factors (nutrition, stress, chemicals, intrauterine milieu, obesity, etc). Epigenetic changes in gene encoding drug-metabolizing enzymes and transporters have been reviewed elsewhere (Cascorbi and Schwab 2016), but their influence on drug response in clinical trials has not been studied.

Conclusions

T2D is a progressive disease with significant variability in the disease course and response to treatment.

Predicting drug response is one of the major challenges of complex diseases management. Disease heterogeneity, genetic variability, gut microbiome activity, and environment contribute to drug response variability. Present guidelines recommend treatment personalization, based only on phenotypic data. Omics-based approaches for diabetes management represent the future. Currently, the genotype is used to guide therapy in the monogenic forms of diabetes. The ability of existing genetic markers to predict drug response is weak to moderate. Further identification of functional variants, gut microbiota, epigenetic modifications, and metabolomics data will further improve the clinical predictability of drug response and reduce the side effects (like hypoglycemia, weight gain, gastrointestinal intolerance). Hopefully, with increasing collaboration internationally, accumulation and availability of big data, we will see omics data translated in clinical practice.

Disclosure statement

The authors have no relevant affiliations or financial involvement.

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Artery Research
Vol. **26**(**2**); June (2020), *pp.* 97–101
DOI: https://doi.org/10.2991/artres.k.200324.001; ISSN 1872-9312; eISSN 1876-4401
https://www.atlantis-press.com/journals/artres

Research Article

Does the Nitric Oxide Synthase *T786C* Gene Polymorphism Influence Arterial Stiffness in Patients with Metabolic Syndrome?

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ARTICLE INFO

Article History
Received 15 March 2019
Accepted 24 March 2020

Keywords
eNOS gene polymorphisms
T786C
arterial stiffness
metabolic syndrome

ABSTRACT

Background: Endothelial Nitric Oxide Synthase (eNOS) is responsible for Nitric Oxide (NO) bioavailability at endothelial level. Aging (even in healthy people) is involved in arterial stiffness increases.

Materials and Methods: We investigated (in the service of Cardiology, 4th Medical Clinic) 100 patients, 55 with metabolic syndrome (MS), mean age 56.91 ± 14.39 years, 66% women. Identification of the *T786C* polymorphism was performed by enzymatic digestion of the fragment obtained by polymerase chain reaction (PCR) amplification. Evaluation of arterial parameters (aortic pulse wave velocity (PWV), as a measure of arterial stiffness and aortic [AixAo] and brachial [Aixb] augmentation index) was performed with the TensioMed™ Arteriograph.

Results: Regarding *T786C* polymorphism, the distribution was the following: 57% did not have the mutation (TT), 30% were heterozygous, 13% were homozygous (CC). Patients with MS more frequently had C allele (54.5% vs. 28.9% in those without MS) and CC state (16.4% vs. 8.9%, *p*-NS). Significant differences ($p = 0.005$) regarding PWV were found in TT patients vs. heterozygous CT vs. homozygous CC: 9.75 ± 1.75 m/s vs. 9.86 ± 1.56 m/s vs. 11.65 ± 1.87 m/s. In case of the other parameters, no significant differences were found (AixAo, $p = 0.35$; Aixb, $p = 0.22$; pulse pressure, $p = 0.14$), but CC patients presented higher values.

Conclusion: Arterial stiffness is influenced by *eNOS* gene polymorphisms, being a possible link between the increase in cardiovascular risk and presence of metabolic syndrome in these patients.

1. INTRODUCTION

An enzyme constitutively expressed mainly in endothelial cells, Endothelial Nitric Oxide Synthase (eNOS or NOS3), is responsible for Nitric Oxide (NO) bioavailability at endothelial level. Alterations in endothelial-derived NO production occurs in various cardiovascular diseases (coronary artery disease, myocardial infarction, hypertension, pre-eclampsia, stroke, metabolic syndrome and diabetes [1–11], associated with different polymorphisms in the *eNOS* gene – one of the most studied being represented by - *786T/C* (rs2070744) [1–11]. Even in relatively healthy people who are at low risk for cardiovascular disease, arterial stiffness increases with advancing age [12].

The interest in studying factors that modulate arterial stiffness is based on the relationship between arterial stiffness and pathogenesis of cardiovascular disease. A cluster entity characterized by hypertension, hyperglycemia, obesity, dyslipidemia, and insulin resistance is known as Metabolic Syndrome (MS). Atherosclerotic lesions in metabolic syndrome can be the result of endothelial dysfunction determined by alteration of nitric oxide production. NO has vasodilatory, antiproliferative and anti-inflammatory effects [13].

2. OBJECTIVES OF THE STUDY

In this study, we aimed to investigate the impact of eNOS *T786C* gene polymorphism on arterial stiffness, by measuring in patients with metabolic syndrome, arterial stiffness parameters -PWV and the augmentation index.

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Peer review under responsibility of the Association for Research into Arterial Structure and Physiology
Data availability statement: The data that support the findings of this study are available from the corresponding author (OHO), upon reasonable request.

3. MATERIALS AND METHODS

3.1. Subjects

One hundred consecutive patients were included in the study, who were investigated in the service of Cardiology, 4th Medical Clinic, University of Cluj-Napoca. Based on International Diabetes Federation (IDF) criteria, 55% of patients were diagnosed with metabolic syndrome (obligatory presence abdominal obesity ≥ 94 cm in men and ≥ 80 cm in women), and another two criteria for blood pressure above 130/85 mmHg, low High-density Lipoprotein (HDL)-cholesterol, glycemia ≥ 100 mg/dl and triglycerides ≥ 150 mg/dl).

Anthropometric measurements were performed and included weight, height and waist circumference. Based on anthropometric measurements, the body mass index was calculated. Blood pressure measurements were performed at least twice, in a quiet room after lying down for 15 min (according to present guidelines) in order to diagnose hypertension.

WHO criteria were used for type 2 diabetes' diagnosis. The levels of Triglycerides (TG), total cholesterol, Low-density Lipoproteins (LDL) and HDLs were estimated according to standard protocols.

The study protocol was approved by the local Ethics Committee, and all subjects provided an oral and written informed consent. The study was conducted in compliance with the Declaration of Helsinki.

3.2. DNA Isolation

The *T786C* polymorphism located in the *eNOS* gene promoter (chr 7q36) was examined by polymerase chain reaction (PCR) amplification of genomic DNA and enzymatic digestion with the restriction endonuclease of the amplified fragment polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP technique).

For the identification of the above mentioned polymorphism, DNA analysis according to the modified method described by Negrao was performed. Amplification was performed in 25 µl reaction mixture, with the following reaction components: 20 ng genomic DNA, 0.2 mM dNTP, 0.2 µM forward and reverse primer [(the forward primer had the sequence: 5′-TGG AGA GTG CTG GTG TAC CCC A-3′; the reverse primer had the sequence: 5′-GCC TCC ACC CCC ACC CTG TC-3′) (Sigma Genosys, The Woodlands, TX 77380, USA), 1.5 mM Mg^{2+} and two units of Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO 63103, USA)]. Identification of the *T786C* polymorphism was performed by enzymatic digestion of the fragment obtained by PCR amplification with the restriction endonuclease MspI. The *T786C* polymorphism creates a restriction site for the MspI enzyme.

3.2.1. Evaluation of arterial parameters

Brachial Augmentation Index (Aixb), Aortic Aix (AixAo), Pulse Wave Velocity (PWVAo), central systolic pressure and aortic Pulse Pressure (PP) was performed with the TensioMed™ Arteriograph (Budapest, Hungary).

3.3. Statistics

Data were statistically processed using the statistical package SPSS 19.0 (IBM Corporation, Armonk, NY, USA) and Medcalc 10.3.0.0 version (MedCalc Software, Ostend, Belgium). The results are presented as mean ± standard deviation for quantitative variables with normal distribution (Kolmogorov test was used for testing the normality of data). Qualitative variables were presented as number (%). The difference between quantitative variables was assessed using the independent-sample *t*-test or Mann–Whitney test, and for qualitative variables, the χ^2-test was used.

Data were presented as odds ratios and 95% confidence intervals. The sensitivity, specificity, positive and negative predictive values, positive likelihood ratio and negative likelihood ratio of a certain mutation/allele in the development of metabolic syndrome were calculated. Univariate analysis and logistic regression was used to identify independent predictive factors for metabolic syndrome. A p-value <0.05 was considered statistically significant.

4. RESULTS

The mean age of the patients included in the study was 56.91 ± 14.39 years, sex distribution being: 66 women and 34 men. There was no significant sex difference regarding the prevalence of MS (53% in women vs. 58.8% in men, p-NS).

Regarding *T786C* polymorphism, the distribution of the 100 subjects was the following: 13% were homozygous (CC), 30% were heterozygous (CT), and 57% of the subjects did not have the mutation (TT). No significant sex differences was found, with the only exception being the absence of the mutation (TT) which was more frequent in the female sex (66.7% vs. 38.2%, p-0.011). The homozygous state (CC) was more frequent in males (20.6% vs. 9.1%, p-NS) compared to females, without a statistically significant difference.

The relationship between the presence of metabolic syndrome and *T786C* polymorphism is presented in Table 1. Homozygous state (CC) was more frequently met in metabolic syndrome patients (CC - 16.4% vs. 8.9%). The TT status was more frequently present in subject without metabolic syndrome (71.1% vs. 45.5% in those with MS, p-0.016).

The C allele was present in a proportion of 54.5% in subjects with metabolic syndrome and 28.9% in controls (p-0.01) – complete data are presented in Table 1.

Prevalence of hypertension and diabetes were increased in homozygous (CC) or heterozygous (CT) state vs. TT patients - 67.4% vs. 40.4%, p-0.0134, for hypertension and 32.6% vs. 10.5%, p-0.0130, for diabetes.

Table 1 | The relationship between metabolic syndrome and the *T786C* mutation

			Metabolic syndrome		Total	*p*
			Yes	No		
T786C mutation	CC	No (%)	9 (16.4)	4 (8.9)	13 (13)	NS
	CT	No (%)	21 (38.2)	9 (20)	30 (30)	0.079
	CC or CT	No (%)	30 (54.6)	13 (28.9)	43 (43)	0.01
	TT	No (%)	25 (45.5)	32 (70.45)	57 (57)	0.021

p between MS patients vs. those without MS, $p < 0.05$ was considered significantly statistic.

The presence of *T786C* gene polymorphism in homozygous (CC) or heterozygous (CT) state was found to be associated with an elevation of glycemia, total cholesterol, LDL-cholesterol, serum triglycerides and a decrease of HDL-cholesterol, with an increased abdominal circumference, but without reaching statistical significance (Table 2).

The eNOS *T786C* gene polymorphism was significantly associated with the presence of metabolic syndrome, subjects having homozygous state had higher risk of having MS compared to those without the polymorphism, OR - 2.790 (95% CI 0.76–10.13, *p*-0.1).

Sensitivity, specificity, positive predictive value, negative predictive value, +LR, –LR of allele presence (CC vs. TT) were calculated (taking into consideration as gold standard for diagnostic IDF criteria for metabolic syndrome definition).

The presence of the C allele was significantly associated with the presence of metabolic syndrome, OR - 2.86 (95% CI 1.2–6.6, *p*-0.014). All data are presented in Tables 1 and 3.

By univariate analysis, we investigated the role of the presence of *T786C* polymorphism in the development of metabolic syndrome. In univariate analysis, increased abdominal circumference, age, weight, elevated glicemia, serum triglycerides, HDL-cholesterol and PWVAo, represent risk factors for the development of MS. The presence of the C allele of the *T786C* mutation was a risk factor for the development of metabolic syndrome (being significantly more frequently present in metabolic syndrome patients 54.5% vs. 28.9%, *p*-0.01). The homozygous state (CC) of *T786C* polymorphism did not represent a risk factor for MS.

Table 2 | The relationship between the *T786C* mutation and biochemical parameters

	Mutation	Mean	Std. dev.	Std. error mean	*p*
Abdominal circumference (cm)	CC/CT	99.32	14.47	2.20	0.07
	TT	93.34	16.37	2.41	
Glycemia (mg/dl)	CC/CT	105.06	28.64	4.36	NS
	TT	97.76	28.07	4.09	
Total cholesterol (mg/dl)	CC/CT	201.39	62.61	9.54	NS
	TT	196.32	50.88	7.76	
LDL-cholesterol (mg/dl)	CC/CT	127.60	49.10	7.96	NS
	TT	119.67	41.14	7.38	
HDL-cholesterol (mg/dl)	CC/CT	41.02	12.62	2.04	NS
	TT	45.00	12.35	2.21	
Triglycerides (mg/dl)	CC/CT	158.11	82.27	12.54	NS
	TT	135.26	69.28	10.69	

Table 3 | Indicators of the risk of *T786C* mutation in homozygous state (CC) compared to the absence of the mutation (TT)

Quantities derived from the two-by-two contingency table	Value	95% Confidence interval	
Odds ratio (OR)	2.790	0.767	10.138
Sensitivity = a/c1 – %	26.47	12.91	44.36
Specificity = d/c2 – %	88.57	73.24	96.7
Positive predictive value (PPV) = a/r1 – %	69.2	38.61	90.72
Negative predictive value (NPV) = d/r2 – %	55.3	41.47	68.65
Positive likelihood ratio (+LR)	2.32	0.79	6.81
Negative likelihood ratio (–LR)	0.83	0.66	1.070

Table 4 | Coefficients and standard errors – logistic regression

	Coefficient	Std. error	*p*
Glycemia	0.122	0.038	0.0014
HDL-cholesterol	–0.207	0.065	0.0016

Using logistic regression, from previous studied factors, backward method (enter variable if $p < 0.05$, remove variable if $p > 0.1$), the independent risk factors for MS were glycemia and HDL-cholesterol. Instead, age, abdominal circumference, weight, triglycerides, PWVAo and C allele were no independent factors, were not included in the model. All data are presented in Table 4.

Regarding *T786C* polymorphism, significant differences of PWV were found between TT vs. CT vs. CC patients: 9.75 ± 1.75 m/s vs. 9.86 ± 1.56 m/s vs. 11.65 ± 1.87 m/s (*p*-0.005) (Table 5).

For the rest of the parameters, only an ascending trend (without statistical significance) was found (AixAo, $p = 0.35$; Aixb, *p*-0.22; PP, $p = 0.14$), with higher values being registered in CC patients.

5. DISCUSSION

The presence of the eNOS *T786C* polymorphism in homozygous or heterozygous state was associated with an increase in the prevalence of arterial hypertension (AHT) and diabetes. Like in this study, Fernandez showed that the *T786C* genotype was significantly more frequent in hypertensive patients with metabolic syndrome compared to those without metabolic syndrome (*p*-0.0022), and concluded that the *eNOS* gene plays an important role in the pathogenesis of metabolic syndrome in hypertensive subjects [14].

Gonzáles-Sánchez et al. [15] reported that the CC genotype was significantly more frequent in subjects with metabolic syndrome compared to those without metabolic syndrome (16.4% vs. 12.5%, *p*-0.010) and in subjects with low HDL cholesterol (16.1% vs. 12.7%, *p*-0.044). These data are in agreement with the data obtained in the current study.

The present study, the *T786C* gene polymorphism was significantly associated with the presence of MS. The eNOS *C786T* gene polymorphism was associated with the presence of metabolic syndrome in hypertensive subjects [14] and the eNOS haplotype, not the G894T polymorphism, was associated with the features of metabolic syndrome.

This inconsistency between studies regarding the association between the *eNOS* gene polymorphism and metabolic syndrome might be explained by genetic heterogeneity and by the difference between environmental factors that influence the phenotypic expression of the mutation [15]. Imamura et al. [16] demonstrated in a study that the *C–786T* allele is associated with increased blood pressure, which is significantly higher compared to subjects without the mutation. Previous studies provide clear evidence of an important physiological role of NO in the modulation of large artery properties [17–19].

Arterial stiffness varies in different arterial districts. In central arteries (such as the aorta) it is strongly influenced by age, elastin and collagen content. However, the tone of vascular smooth muscle through NO, influences the stiffness of the medium-sized muscular arteries.

Table 5 | Arterial parameters depending on the *T786C* mutation

Arterial parameters	*T786C* mutation	Mean	Std. dev.	Std. error	95% CI for mean		Min. values	Max. values	*p*
					Lower	Upper			
PWVAo	TT	9.75	1.75	0.30	9.13	10.38	5.80	14.10	0.005
	CT	9.86	1.56	0.32	9.19	10.54	7.70	13.10	
	CC	11.65	1.87	0.54	10.46	12.84	8.80	14.60	
Aixb	TT	−2.07	31.96	5.48	−13.23	9.07	−59.60	52.50	0.22
	CT	1.30	26.85	5.48	−10.03	12.63	−68.30	54.60	
	CC	15.68	31.33	9.04	−4.22	35.59	−47.90	55.60	
AixAo	TT	34.46	18.11	3.06	28.24	40.68	0.00	64.20	0.35
	CT	37.97	17.24	3.52	30.69	45.26	0.00	72.10	
	CC	42.71	15.24	4.40	33.03	52.40	13.40	65.80	
PP	TT	53.37	12.81	2.16	48.96	57.77	36.00	80.00	0.14
	CT	51.33	11.76	2.40	46.36	56.30	35.00	77.00	
	CC	60.91	19.42	5.60	48.57	73.25	39.00	100.00	

p between registered values in TT patients vs. CT patients vs. CC patients (*p* was calculated using ANOVA test).

Endothelium-derived NO (synthesized from L-arginine by eNOS [20–22] has multiple physiological properties, modulating growth and migration of vascular smooth muscle cells and relaxing vascular smooth muscle [20,23]. Genetic factors, determining eNOS abnormalities, reduce bioavailability of NO [21,23].

In previous studies on mice, NOS3 knockout was associated with elevation in pulse pressure [21]. NOS3 has a role in the modulation of arterial properties, an association between NOS3 gene polymorphism and arterial function being found. In several studies, *eNOS* gene polymorphisms have been associated with arterial stiffness parameters [22,24].

Using ANOVA test, we found significant differences regarding PWVAo between TT and CT and CC subjects. For Aixb, AixAo and aortic PP, homozygous (CC) patients presenting higher values, but without statistical significance. Mayer et al. [22] demonstrated in a study that the homozygous and heterozygous status of *T786C* polymorphism is accompanied by significantly higher values of pulse wave velocity compared to mutation-free subjects (14.0 vs. 10.7 m/s, $p < 0.002$); Mitchell showed that Glu298Asp polymorphism is correlated with pulse pressure and the reflected wave amplitude only in women [23]. After adjustment for multiple factors, the association between eNOS polymorphism and arterial stiffness was no longer maintained. Our group found that *G894T* polymorphism did not significantly influence the values of the arterial stiffness (PWV, Aixb and AixAo) [24]. The mutated T allele of rs3918226 polymorphism in the *NOS3* gene was associated with parameters reflecting central arterial stiffness and wave reflection [25].

In women but not men, the genotype for the common NOS3 missense mutation (Glu298Asp, rs1799983) was related to central pulse pressure and forward wave amplitude [23]. In the current study, in univariate analysis, age, weight, increased abdominal circumference, elevated levels of glycemia and serum triglycerides, low HDL-cholesterol and high PWVAo represented risk factors for the development of metabolic syndrome.

The presence of the C allele of the *T786C* mutation was a risk factor for the development of metabolic syndrome.

6. CONCLUSION

The eNOS *T786C* gene polymorphism in homozygous and heterozygous state was significantly associated with the presence of metabolic syndrome and with an increase in the prevalence of AHT and diabetes mellitus.

The *T786C* polymorphism influences arterial stiffness parameters, specifically PWV, which is the gold standard for arterial stiffness. Arterial stiffness is influenced by eNOS *T786C* gene polymorphisms, being a possible link between the increase cardiovascular risk and presence of metabolic syndrome in these patients.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

AUTHORS' CONTRIBUTION

AC, AV and ST contributed in concept and design of the study, data acquisition, analysis and interpretation of data, drafting and revising the article for intellectual content. OHO contributed in concept and design of the study, analysis and interpretation of data, drafting and revising the article for intellectual content. AF contributed in data acquisition, analysis and interpretation of data, drafting and revising the article. TA and VN contributed in analysis and interpretation of data, revising the article for intellectual content. LMP contributed in genetic analysis and interpretation of data, drafting and revising the article. DP and DAT contributed in concept and design of the study, revising the article for intellectual content. All authors read and approved the final manuscript.

FUNDING

No financial support was provided.

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METABOLIC SYNDROME AND RELATED DISORDERS
Volume XX, Number XX, 2020

Pp. 1–7
DOI: 10.1089/met.2020.0056

The Impact of Obesity on Left Ventricular Hypertrophy and Diastolic Function in Caucasian Children

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Abstract

Background: Left ventricular hypertrophy (LVH) and diastolic dysfunction are correlated with obesity and hypertension in adult patients, but few studies have investigated the association between obesity itself and left ventricular function in children. The aim of this study was to evaluate the effect of obesity and LVH on left ventricular diastolic function in pediatric subjects compared with children without obesity.
Methods: A number of 454 patients from an outpatient cardiology service were enrolled in a prospective study, 33 children with obesity, 20 overweight children, and 401 children without obesity. The subjects were assigned to three groups according to age and school grade. A standardized two-dimensional echocardiography analysis was performed in all children. The evaluated echocardiographic parameters included thickness of the interventricular septum (IVS), thickness of the posterior wall of the left ventricle, and left atrium size. The left ventricular diastolic function was analyzed by the classic pulsed-wave Doppler technique, tissue Doppler technique, and continuous Doppler technique.
Results: The number of children with obesity was higher in the school and adolescent groups. The median age of children with obesity was 9 years. The subjects were classified according to blood pressure values in hypertensive, with high–normal blood pressure/prehypertension and with normal blood pressure values. Standard echocardiography showed that children with obesity had significantly increased thickness of the IVS and of the posterior wall compared with nonobesity subjects ($P < 0.001$). Left ventricular systolic function was preserved in both groups. Diastolic function was normal in the obesity group and in the non-obesity group, respectively.
Conclusions: The results of this study demonstrate that childhood obesity is associated with significant changes in the myocardial structure consisting of LVH, but we did not find an early alteration in the left ventricular diastolic function of the subjects with obesity compared with patients with a normal weight.

Keywords: left ventricular hypertrophy, diastolic function, obesity, body mass index, children, blood pressure

Introduction

OBESITY AMONG CHILDREN and adolescents is becoming an increasingly common health problem. The prevalence of obesity among the population aged 2–19 years in the United States is 18.5% (13.7 million children and adolescents).[1] Furthermore, in Europe, data on the prevalence of overweight and obesity among primary school children are not encouraging. Studies show that severe obesity affects 400,000 of the 21.7 million people aged 6–9 years from the 21 countries participating in the study.[2] Worrisome is the fact that overweight and obesity during childhood have been associated with cardiovascular disease and premature death in later life.[3]

Clinical studies have identified a number of changes in the heart geometry[4] based on data from imaging investigations, such as echocardiography, including increased left ventricular mass and ventricular hypertrophy,[5] parameters strongly associated with an increased risk of cardiovascular disease, and premature death among adults.[3] Other changes

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identified among overweight children and children with obesity are high blood pressure, hypercholesterolemia, and insulin resistance, which are all associated with adverse cardiovascular events.[6]

There are studies showing that obesity and hypertension are independent factors in terms of left ventricular hypertrophy (LVH). The blood volume load in patients with obesity is consistently higher than in normal weight patients, even in the presence of blood pressure levels within the normal range.[7] Volume overload causes compensatory changes in cardiac geometry, the consequence of being a maladaptive change in a structure characterized by eccentric LVH, whereas the result of pressure overload is concentric LVH.[8] It is important to mention that in the case of athletes, LVH represents a physiologically adaptive mechanism of the heart depending on the type of sport performed: endurance or strength training.[9]

Objective

The main purpose of this study was to assess the prevalence of ventricular hypertrophy among the pediatric population with obesity. For this purpose, cardiac parameters were evaluated using standard echocardiography, pulsed-wave Doppler technique, tissue Doppler technique, and continuous Doppler technique both in children with obesity and in children with normal weight. Furthermore, we investigated other clinical factors that could influence the left ventricular mass such as anthropometric factors, blood pressure, and biochemical parameters including serum total cholesterol levels.

Materials and Methods

Patients

This study was carried out based on echocardiographic data collected from 454 children and adolescents, 234 boys and 220 girls, aged 0–17 years, of whom 33 had obesity (20 boys and 13 girls), 20 were overweight (9 boys and 11 girls), and 401 children had a normal weight (205 boys and 196 girls). All children underwent the aforementioned procedure due to cardiac murmurs detected by the general practitioner during routine examinations, dyspnea, heart palpitations, syncope or angina, or just because of parents' request.

The subjects were assigned to three groups according to age and school grade: 268 were aged 0 to pre-school (0–6 years old, of whom 11 had obesity, 9 were overweight), 117 school children (7–11 years old, 14 had obesity, 6 were overweight), and 69 adolescents (12–17 years old, 8 had obesity, 5 were overweight). None of the patients with obesity included in the study had a previous diagnosis of arrhythmia, congenital heart defects, or pulmonary hypertension.

An informed consent was signed by the legal guardians of all children included in the study, expressing their agreement to participate in this study. The study protocol was approved by the local ethics committee.

Echocardiographic measurements

A thorough echocardiographic evaluation of each patient included in our study was performed. For this purpose, we used a General Electric echocardiography device, Vivid 6S model. The windows and views used to collect the necessary data for the study were the standard windows for echocardiography in children, which include: parasternal views, apical views, subcostal views, and suprasternal views.[10] For the assessment of the heart chambers, wall thickness, ventricular function, and valve function, a standardized two-dimensional echocardiography analysis was performed in all children. The echocardiographic parameters used in our study were thickness of the interventricular septum (IVS), thickness of the posterior wall of the left ventricle (PWLV), and left atrium size.

The left ventricular diastolic function was analyzed according to the current guidelines using the classic pulsed-wave Doppler technique (mitral flow, pulmonary vein flow), tissue Doppler technique, and continuous Doppler technique (tricuspid regurgitation systolic jet velocity).

All children were examined by the same cardiologist in left lateral position, in a dark room. The evaluations of left ventricular changes were interpreted using the percentile values of left ventricular measurements for age and gender.[11]

Laboratory procedures

Laboratory tests were performed in each patient included in our study. We determined the plasma level of total cholesterol.

Anthropometric measurements and demographic data

All subjects in this study were Caucasians. Data regarding sex and age were collected for each patient.

To define obesity and overweight, the Centers for Disease Control and Prevention (CDC) growth charts were used for children and adolescents aged between 2 and 17 years.

Body mass index (BMI) for age and sex growth charts was utilized to establish the presence of overweight/obesity. The children aged between 2 and 17 years were classified as follows: obesity group BMI for age ≥95th percentile, overweight group BMI for age ≥85th and <95th percentile, normal weight ≥5th and <85th percentile, and underweight group <5th percentile.[12] For the subjects aged between 0 and 2 years, the World Health Organization weight-for-length charts were used, and the subjects were classified as follows: obesity group (high weight for length group) >98th percentile, underweight group (low weight for length group) <2nd percentile, and the normal weight group (normal weight for length group) between 2nd and 98th percentile.[13]

The office systolic blood pressure and diastolic blood pressure were measured after a 5 min rest, in sitting position, using an appropriate size blood pressure cuff. The blood pressure data were expressed as percentiles by sex, age, and height or length for children aged between 1 and 17 years, and the subjects were classified as follows: the grade I hypertension group between 95th and 99th percentile, group with normal high blood pressure/prehypertension between 90th and 95th percentile, and normal blood pressure group between 50th percentile and 90th percentile.[14] For infants and neonates, the Pediatric Intensive Care Unit (PICU) chart was used to evaluate the blood pressure values.[15]

Electrocardiogram data

Each subject included in the study underwent an electrocardiogram procedure to identify any indications suggestive of LVH or arrhythmias.

Statistical analysis

Statistical analysis was performed using the statistical packages SPSS 19.0 (IBM Corporation, Armonk, NY) and MedCalc version 10.3.0.0 (MedCalc Software, Ostend, Belgium). Qualitative variables are presented as absolute values and percentages. After testing quantitative variables for normal distribution (using the Kolmogorov test), these are reported as mean ± standard deviation and median value, respectively. The chi-squared test, independent-sample *t*-test, or Mann–Whitney test were used to assess differences between variables. A P value <0.05 was considered statistically significant.

Results

A total of 454 patients, aged 0–17 years, all Caucasians, were included in our study.

Table 1. Patients' Characteristics

	Global *454 patients*	*Boys* *234*	*Girls* *220*	P
Age, years, *n* (%)				
0–6	268 (59)	140 (59.83)	128 (58.18)	ns
7–11	117 (25.8)	56 (23.93)	61 (27.73)	
12–17	69 (15.2)	38 (16.24)	31 (14.09)	
Age,[a] years				
Mean ± SD (median)	5.97 ± 4.77 (5)	5.94 ± 4.78 (5)	6.00 ± 4.78 (5)	ns
Obesity				
n (%)—yes	33 (7.3 of all patients)	20 (8.54 of all boys)	13 (5.9 of all girls)	
0–6	11 (33.33 of patients with obesity)	6 (30 of patients with obesity)	5 (38.46 of patients with obesity)	ns
7–11	14 (42.42)	8 (40)	6 (46.15)	
12–17	8 (24.24)	6 (30)	2 (15.38)	
Overweight				
n (%)—yes	20 (4.4 of all patients)	9 (3.84 of all boys)	11 (5 of all girls)	
0–6	9 (45 of overweight patients)	5 (55.55 of overweight patients)	4 (36.36 of overweight patients)	ns
7–11	6 (30)	1 (11.11)	5 (45.45)	
12–17	5 (25)	3 (33.33)	2 (18.18)	
Hypertension				
n (%)—yes	30 (6.6 of all patients)	18 (7.69 of all boys)	12 (5.45 of all girls)	ns
0–6	20 (66.66 of hypertensive)	12 (66.66 of hypertensive)	8 (66.66)	—
7–11	3 (10)	1 (5.55)	2 (16.66)	
12–17	7 (23.33)	5 (27.77)	2 (16.66)	
Prehypertension normal high blood pressure				
n (%)—yes	73 (16.07 of all patients)	37 (15.81 of all boys)	36 (16.36 of all girls)	ns
0–6	51 (69.86 of prehypertensive/ normal high)	24 (64.86 of prehypertensive/ normal high)	27 (75 of prehypertensive/ normal high)	
7–11	7 (9.58)	3 (8.1)	4 (11.11)	
12–17	15 (20.54)	10 (27.02)	5 (13.88)	
Diabetes				
n (%)—yes	4 (0.88 of all patients)	1 (0.42 of all patients)	3 (1.36 of all patients)	ns
0–6	1 (25 of all diabetes)	0 (0)	1 (33.33)	
7–11	0 (0)	0 (0)	0 (0)	
12–17	3 (75)	1 (100)	2 (66.66)	
IVS[a]				
Mean ± SD (median)	5.9 ± 2.14 (5.5)	5.93 ± 1.74 (5.5)	5.87 ± 2.49 (5.5)	ns
0–6	5.07 ± 2.11 (5)	5.06 ± 1.11 (5)	5.09 ± 2.83 (5)	ns
7–11	6.51 ± 1.23 (6)	6.48 ± 1.35 (6.25)	6.54 ± 1.12 (6)	ns
12–17	8.09 ± 1.46 (8)	8.37 ± 1.52 (8.4)	7.75 ± 1.34 (8)	0.055
PWLV[a]				
Mean ± SD (median)	5.94 ± 1.63 (6)	6.02 ± 1.76 (6)	5.85 ± 1.49 (6)	ns
0–6	5.02 ± 1.04 (5)	5.08 ± 1.13 (5)	4.96 ± 0.94 (5)	ns
7–11	6.68 ± 1.11 (6)	6.68 ± 1.23 (6)	6.68 ± 1.00 (6)	ns
12–17	8.24 ± 1.34 (8)	8.51 ± 1.44 (9)	7.91 ± 1.14 (8)	0.033

[a]Does not respect normal distribution.
IVS, interventricular septum; ns, not statistically significant; PWLV, posterior wall of left ventricle; SD, standard deviation.

The subjects were assigned to three groups according to age and school grade: 268 (59%) patients were aged between 0 and 6 years, 117 (25.8%) school children were aged between 7 and 11 years, and 69 (15.2%) patients were adolescents (12–17 years old).

The gender distribution in this study was 234 (51.5%) boys and 220 (48.5%) girls. The global mean age was 5.97 ± 4.77 (median value 5) years, with no significant difference between boys and girls.

Of all children included in the study, 7.26% (33 subjects) had obesity: 13 of these were girls (5.9% of all girls) and 20 were boys (8.54% of all boys); 4.4% (20 subjects) were overweight: 11 of these were girls (5% of all girls) and 9 were boys (3.84% of all boys). The number of children with obesity was higher in school (11.96%) and adolescent (11.59%) groups. The mean age of children with obesity was 8.66 ± 3.53 (median value 9) years, and of children with overweight 7.58 ± 5.84 (median value 7) years.

Blood pressure measurements were performed in all children; 103 (22.7%) had high–normal blood pressure/prehypertension [73 patients (16.1%)] or hypertension [30 patients (6.6%)]; 351 (77.3%) had normal blood pressure values, with no significant differences between genders. Two (0.5%) children with high serum total cholesterol levels were identified. Four (0.88%) patients included in the study had been previously diagnosed with type 1 diabetes (3 girls and 1 boy).

All data regarding patients' characteristics are presented in Table 1.

Standard echocardiography showed for the IVS mean value of 5.9 ± 2.14 (median value 5.5) mm and for the PWLV mean value of 5.94 ± 1.63 (median value 6), with no significant difference between boys and girls. Further analysis evidenced significant differences between boys and girls in the 12–17 years age group [for IVS 8.37 ± 1.52 (median value 8.4) vs. 7.75 ± 1.34 (median value 8), $P = 0.055$ at limit, and for PWLV 8.51 ± 1.44 (median value 9) vs. 7.91 ± 1.14 (median value 8), $P = 0.0033$].

Following the analysis of the entire group, significant differences were found between obesity and nonobesity/nonoverweight patients regarding IVS [7.55 ± 1.59 (median value 7) vs. 5.72 ± 2.12 (median value 5), $P < 0.0001$] at the general level and in both boys [7.44 ± 1.65 (median value 7.25) vs. 5.74 ± 1.63 (median value 5), $P < 0.0001$] and girls [7.73 ± 1.54 (median value 7) vs. 5.7 ± 2.53 (median value 5), $P < 0.0001$].

The same relationship was found for PWLV, differences being significant between obesity and nonobesity patients of both genders [boys 7.6 ± 1.51 (median value 8) vs. 5.8 ± 1.66 (median value 6), $P < 0.0001$, and girls 7.8 ± 1.42 (median value 7.5) vs. 5.66 ± 1.38 (median value 5.5), $P < 0.0001$]. All detailed data are presented in Tables 2–4.

Left ventricular systolic function was preserved in both groups. Diastolic function was normal in the obesity group and in the nonobesity group, respectively.

Discussion

In this study, we investigated the relationship between obesity and cardiac geometry, independent of hypertension. Our data demonstrated that normotensive children with obesity present early asymptomatic changes in a myocardial structure consisting of LVH. We found significant

Table 2. Differences Between Patients with Obesity Versus Patients Without Obesity at Global Level

	Obesity				*Nonobesity/nonoverweight*				
	Global 33 patients	*HBP 5 patients*	*Pre-HBP/NH 3 patients*	*No HBP 25 patients*	*Global 401 patients*	*HBP 24 patients*	*Pre-HBP/NH 66 patients*	*No HBP 311 patients*	P[a]
All									
IVS	7.55 ± 1.59 (7)	7.4 ± 1.67 (7)	7.33 ± 1.44 (6.5)	7.61 ± 1.65 (7.5)	5.72 ± 2.12 (5)	5.7 ± 1.39 (5)	5.92 ± 1.52 (5.5)	5.68 ± 2.27 (5)	<0.0001
PWLV	7.68 ± 1.46 (7.5)	7.6 ± 1.34 (7)	7.66 ± 1.15 (7)	7.7 ± 1.56 (8)	5.74 ± 1.53 (6)	5.72 ± 1.48 (5)	6.02 ± 1.54 (6)	5.69 ± 1.53 (6)	<0.0001
0–6 Years									
IVS	6.63 ± 1.16 (6.5)	6 ± 1.41 (6)	6.5 (6.5)	6.85 ± 1.31 (7)	5.00 ± 2.15 (5)	5 ± 0.59 (5)	5.36 ± 1.03 (5)	4.9 ± 2.44 (5)	<0.0001
PWLV	6.81 ± 0.87 (7)	6.5 ± 0.7 (6.5)	7 (7)	6.85 ± 1.06 (6.5)	4.93 ± 0.99 (5)	5.02 ± 0.49 (5)	5.42 ± 1.06 (5)	4.79 ± 0.97 (5)	<0.0001
7–11 Years									
IVS	7.39 ± 1.34 (7)	—	9 (9)	7.26 ± 1.31 (7)	6.35 ± 1.18 (6)	7.66 ± 0.57 (8)	5.9 ± 0.54 (6)	6.33 ± 1.2 (6)	0.0035
PWLV	7.67 ± 1.42 (7.75)	—	9 (9)	7.57 ± 1.42 (7.5)	6.49 ± 0.99 (6)	7.33 ± 2.08 (8)	6.2 ± 0.44 (6)	6.48 ± 0.96 (6)	0.0017
12–17 Years									
IVS	9.1 ± 1.48 (8.9)	8.33 ± 1.15 (9)	—	9.56 ± 1.57 (8.8)	7.83 ± 1.38 (8)	8 ± 1 (8)	8.2 ± 1.37 (8)	7.71 ± 1.41 (8)	0.0489
PWLV	8.87 ± 1.45 (8.5)	8.33 ± 1.15 (9)	—	9.2 ± 1.64 (8)	8.05 ± 1.28 (8)	8.33 ± 0.57 (8)	8.37 ± 1.18 (8)	7.93 ± 1.34 (8)	0.1675

Values are presented as mean ± SD (median).
[a]Between obesity and nonobesity patients at global level.
HBP, high blood pressure/hypertension; Pre-HBP/NH, prehypertension or normal high.

Table 3. Differences Between Obesity Versus Nonobesity Patients in Boys

	Obesity				*Nonobesity/nonoverweight*				
	Global 20 patients	*HBP 5 patients*	*Pre-HBP/NH 1 patient*	*No HBP 14 patients*	*Global 205 patients*	*HBP 12 patients*	*Pre-HBP/NH 35 patients*	*No HBP 158 patients*	P[a]
All									
IVS	7.44±1.65 (7.25)	7.4±1.67 (7)	9±0 (9)	7.34±1.71 (7.25)	5.74±1.63 (5)	5.25±0.96 (5)	6.3±1.77 (5.5)	5.66±1.62 (5)	<0.0001
PWLV	7.6±1.51 (8)	7.6±1.34 (7)	9±0 (9)	7.5±1.62 (8)	5.82±1.66 (6)	5.45±1.23 (5)	6.42±1.75 (6)	5.71±1.65 (6)	<0.0001
0–6 Years									
IVS	6.33±1.5 (6)	6±1.41 (6)	—	6.5±1.73 (6)	4.99±1.08 (5)	4.9±0.56 (5)	5.45±1.13 (5)	4.89±1.08 (5)	0.0188
PWLV	6.5±1 (6.5)	6.5±0.7 (6.5)	—	6.5±1.22 (6.5)	5.0±1.11 (5)	4.95±0.36 (5)	5.58±1.13 (5.5)	4.86±1.11 (5)	0.0025
7–11 Years									
IVS	7.25±1.36 (7.25)	—	9±0 (9)	7±1.25 (7)	6.34±1.33 (6)	7±0 (7)	6.25±0.35 (6.25)	6.32±1.37 (6)	0.0389
PWLV	7.62±1.48 (8.25)	—	9±0 (9)	7.42±1.48 (8)	6.52±1.14 (6)	8±0 (8)	6.5±0.7 (6.5)	6.48±1.15 (6)	0.0317
12–17 Years									
IVS	8.8±1.32 (8.9)	8.33±1.15 (9)	—	9.26±1.55 (8.8)	8.12±1.5 (8)	7±0 (7)	8.55±1.33 (9)	7.97±1.6 (8)	0.4181
PWLV	8.66±1.36 (8.5)	8.33±1.15 (9)	—	9±1.73 (8)	8.32±1.44 (8.5)	8±0 (8)	8.66±1.22 (9)	8.18±1.57 (8.5)	0.7928

Values are presented as mean±SD (median).
[a]Between obesity and nonobesity patients at global level.

Table 4. Differences Between Obesity Versus Nonobesity Patients in Girls

	Obesity				*Nonobesity/nonoverweight*				
	Global 13 patients	*HBP 0 patients*	*Pre-HBP/NH 2 patients*	*No HBP 11 patients*	*Global 196 patients*	*HBP 12 patients*	*Pre-HBP/NH 31 patients*	*No HBP 153 patients*	P[a]
All									
IVS	7.73±1.54 (7)	—	6.5±0 (6.5)	7.95±1.58 (7.5)	5.7±2.53 (5)	6.16±1.64 (5.5)	5.5±1.07 (5.5)	5.7±2.79 (5)	<0.0001
PWLV	7.8±1.42 (7.5)	—	7±0 (7)	7.95±1.5 (7.5)	5.66±1.38 (5.5)	6±1.7 (5)	5.5±1.11 (6)	5.66±1.41 (5.5)	<0.0001
0–6 Years									
IVS	7±0.5 (7)	—	6.5±0 (6.5)	7.33±0.28 (7.5)	5.0±2.9 (5)	5.12±0.64 (5)	5.28±0.95 (5.5)	4.91±3.37 (5)	0.0003
PWLV	7.2±0.57 (7)	—	7±0 (7)	7.33±0.76 (7.5)	4.85±0.84 (5)	5.12±0.64 (5)	5.28±0.97 (5)	4.71±0.77 (5)	0.0002
7–11 Years									
IVS	7.58±1.42 (7)	—	—	7.58±1.42 (7)	6.37±1.04 (6)	8±0 (8)	5.66±0.57 (6)	6.34±1.02 (6)	0.0298
PWLV	7.75±1.47 (7.25)	—	—	7.75±1.47 (7.25)	6.47±0.84 (6)	7±2.82 (7)	6±0 (6)	6.47±0.76 (6)	0.0235
12–17 Years									
IVS	10±2.12 (10)	—	—	10±2.12 (10)	7.53±1.18 (7.5)	8.5±0.7 (8.5)	7.16±1.04 (7.5)	7.5±1.22 (7.25)	0.0707
PWLV	9.5±2.12 (9.5)	—	—	9.5±2.12 (9.5)	7.75±1.04 (7.5)	8.5±0.7 (8.5)	7.5±0.5 (7.5)	7.72±1.10 (7.5)	0.1320

Values are presented as mean±SD (median).
[a]Between obesity and nonobesity patients at global level.

differences between children with and without obesity regarding PWLV and IVS. These alterations were present in both girls and boys. The left ventricular systolic function was preserved in both groups. Also, we did not find changes in the diastolic function of the children with or without obesity. In our study, we included Caucasian children from Romania; therefore, our article accurately reflects the situation in our country. The prevalence of obesity and overweight in the pediatric population is strongly influenced by local eating habits and lifestyle.

Co-morbidities associated with obesity can cause significant damage to the cardiovascular system.[16] The purpose of our study was to demonstrate that obesity itself has a significant impact on cardiac geometry, given that children generally have no co-morbidities. We were able to demonstrate that overweight children have IVS and posterior wall hypertrophy compared with nonobesity patients. We found no changes in cardiac diastolic function, but the present study focused on resting cardiac function. It is possible that the evaluation performed under stress conditions could provide other results regarding diastolic function, being even able to detect early cardiac dysfunction in the case of subjects with obesity and cardiac function within the normal range in resting conditions.[17]

The interpretation of cardiac parameters in children is challenging, given that they must be related to physical parameters such as age, BMI, sex, height, and lean body mass.[18] In our study, we evaluated cardiac size in relation to the patients' BMI, sex, and age.

Factors associated with changes in cardiac geometry in children with obesity include, in addition to hypertension (HT), increased cardiac output,[19] local effects of the renin–angiotensin–aldosterone system, and insulin resistance syndrome.[20]

Since studies are showing that childhood obesity may be a risk factor for cardiovascular disease in adulthood,[21] early diagnosis of heart changes in children with obesity and their long-term follow-up are crucial.

It is important to mention the fact that some studies demonstrated the beneficial effects of weight loss on changes in cardiac geometry. In addition, the most important predictor of left ventricular mass index decrease was a reduction in abdominal fat expressed as a decrease in waist circumference.[22] Moreover, studies conducted in treated hypertensive adult patients show that residual LVH is much more common in subjects with obesity than in normal weight individuals,[23] which further emphasizes the importance of weight loss simultaneously with antihypertensive treatment.

In daily clinical practice, it is important to advise children and their families on the importance of weight loss and embracing a healthy lifestyle together with regular outdoor physical activity.

Study limitations

Our study has some limitations. First of all, the current study included only Caucasian subjects, so our results cannot be extrapolated to other populations. Also, we did not evaluate the effect of other cardiometabolic risk factors, such as fasting blood glucose, insulinemia, and the presence of systemic inflammation in the assessment of cardiovascular risk.

Another shortcoming of this study is the small sample of subjects with obesity in relation to normal weight children. Also, the age of the subjects included in the study may have influenced the results, children included in our study having an average age (9 years), which is much lower than the mean age of the subjects included in other studies (11 and 14 years, respectively).

Our results are also based on examinations performed in resting conditions. Stress examinations might have identified changes that we could have missed in our investigation.

In our study, we used office blood pressure measurements, which do not match the quality of ambulatory blood pressure measurements.

Conclusions

The results of this study demonstrate that childhood obesity is associated with significant changes in myocardial structure consisting of LVH, but we did not find an early alteration in the left ventricular diastolic function of subjects with obesity compared with nonobesity patients. All these suggest that the adverse effects of obesity on the cardiovascular system have an early onset and emphasize the importance of identifying these changes and treating them to prevent their consequences in adult life.

Author Disclosure Statement

No conflicting financial interests exist.

Funding Information

The authors received no funding for this research article.

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International Journal of
Molecular Sciences

MDPI

Review

MicroRNAs: The Link between the Metabolic Syndrome and Oncogenesis

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Abstract: Metabolic syndrome (MetS) represents a cluster of disorders that increase the risk of a plethora of conditions, in particular type two diabetes, cardiovascular diseases, and certain types of cancers. MetS is a complex entity characterized by a chronic inflammatory state that implies dysregulations of adipokins and proinflammatory cytokins together with hormonal and growth factors imbalances. Of great interest is the implication of microRNA (miRNA, miR), non-coding RNA, in cancer genesis, progression, and metastasis. The adipose tissue serves as an important source of miRs, which represent a novel class of adipokines, that play a crucial role in carcinogenesis. Altered miRs secretion in the adipose tissue, in the context of MetS, might explain their implication in the oncogenesis. The interplay between miRs expressed in adipose tissue, their dysregulation and cancer pathogenesis are still intriguing, taking into consideration the fact that miRNAs show both carcinogenic and tumor suppressor effects. The aim of our review was to discuss the latest publications concerning the implication of miRs dysregulation in MetS and their significance in tumoral signaling pathways. Furthermore, we emphasized the role of miRNAs as potential target therapies and their implication in cancer progression and metastasis.

Keywords: metabolic syndrome; adipose tissue; miRNA; cancer; metastases

Citation: Fodor, A.; Lazar, A.L.; Buchman, C.; Tiperciuc, B.; Orasan, O.H.; Cozma, A. MicroRNAs: The Link between the Metabolic Syndrome and Oncogenesis. *Int. J. Mol. Sci.* **2021**, *22*, 6337. https://doi.org/10.3390/ijms22126337

Academic Editors: Elisabetta Ferretti, Zein Mersini Besharat and Agnese Po

Received: 1 May 2021
Accepted: 9 June 2021
Published: 13 June 2021

1. Introduction

Metabolic syndrome is a cluster of at least three pathophysiological disorders: abdominal obesity, low-high density lipoprotein (HDL) cholesterol levels, high triglycerides levels, hypertension, and hyperglycemia [1]. The worldwide prevalence varies between 10 and 40%, depending on lifestyle and genetic background [2]. Diet, lifestyle, and genetic background not only affect MetS, but there is increasing evidence showing that these factors play a crucial role in tumorigenesis. MetS has become a predominant risk factor for many cancer types.

Abdominal obesity represents the central feature of MetS and it is associated with alterations in immunity and chronic low-grade inflammation. These can lead to the dysfunction of adipose tissue homeostasis, insulin resistance (IR), macrophages infiltration and polarization, and the release of inflammatory cytokines from both adipose and immune cells. Macrophages are the main immune cells contributing to the activation of inflammatory pathways in obesity and other metabolic conditions. The metabolic and

inflammatory changes in adipose tissue can disrupt physiological homeostasis systemically, with initiation and progression of metabolic syndrome and cancer.

Evidence of the last decade supports the crucial role of inflammation in tumor development, progression, and immunosuppression. Most cancers are associated with chronic inflammation induced by environmental factors, such as asbestos, tobacco smoking, and dietary factors, or by chronic bacterial and viral infections, like cervical carcinoma, liver and gastric cancer [3]. Inflammation has been recognized as an essential tumorigenic factor, but it is also often present in the microenvironment of cancers without inflammatory origins [3]. Obesity produces chronic inflammation and an altered profile of key transcription factors that promote a procarcinogen microenvironment. People with obesity have a higher risk of many types of cancers, such as esophagus, gastric, colon, rectum, prostate, liver, prostate, kidney, ovary, meningioma, multiple myeloma, thyroid [4].

Adipose tissue is a complex endocrine organ secreting not only hormones and cytokines, but also a large number of circulating miRs. Adipose tissue derived-miRs from people with obesity have proved essential role not only in obesity-associated inflammation and IR, but in tumor growth and metastasis. Specific miR may act both as miR oncogenic (oncomiR) by suppressing tumor suppressive mRNAs and tumor-suppressive molecule, by suppressing oncogenic mRNAs.

This systematic literature review aimed to revise recent publications on the role of obesity or MetS microRNAs deregulation in adverse prognosis of cancer. Furthermore, we highlight the potential therapeutic role of adipose tissue-derived miRNAs.

We reviewed all publications from the PubMed database using the terms:

(((((“obesity”[MeSH Terms]) OR (“insulin resistance”[MeSH Terms])) OR (“metabolic syndrome”[MeSH Terms])) AND (“neoplasms”[MeSH Major Topic]))) AND (micrornas-[MeSH Terms]).

Each relevant microRNA was included in the present analysis if it fulfilled both searches:

1. (“neoplasm invasiveness/etiology”[MeSH Terms]) AND specific microRNA,
2. ((“obesity”[MeSH Terms]) OR (“insulin resistance”[MeSH Terms])) OR (“metabolic syndrome”[MeSH Terms]) AND specific microRNA.

2. MicroRNAs Link the Metabolic Syndrome and Cancer

MicroRNAs (miRs) are small (about 18–25 nucleotides), non-coding RNAs, which negatively regulate gene expression by translational inhibition or mRNA decay. miR is associated with Argonaut proteins and incorporated into the miR-induced silencing complex (mRISC), which guides the binding of miRs to the 3′UTR of the target mRNAs. Due to short binding sequence, with imperfect complementary, an individual miR can bind and affect the expression of hundreds of mRNAs [5]. In addition, miRs have been found to be secreted to the extracellular space as membrane-covered microvesicles, such as exosomes, which can be taken up by neighboring or distant recipient cells. Adipocyte-derived microvesicles contain about 140 miRs, while in adipose tissue, macrophages-derived exosomes are identified in about 500 miRs [6,7]. Several pathways, controlled by miRs, have been proposed in the last decade to explain the increased risk of cancer in the context of MetS. We emphasize below the most relevant ones (see also Figure 1).

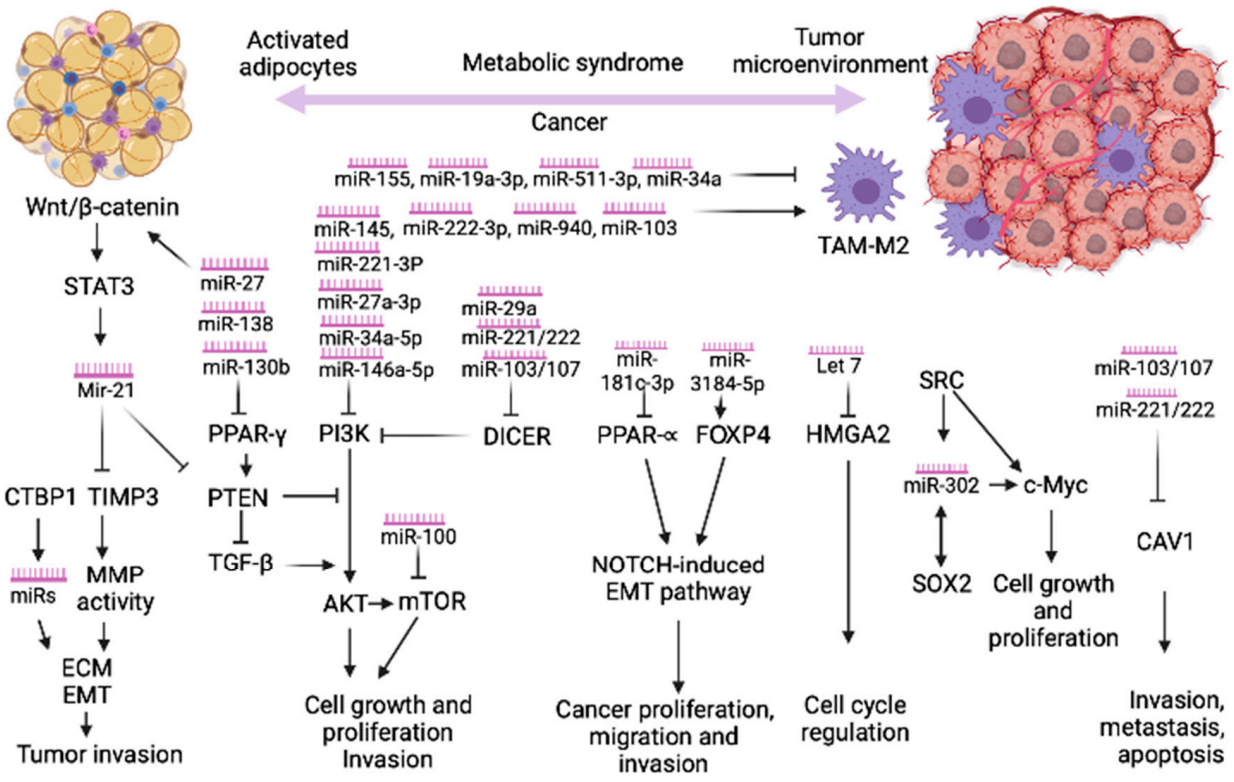

Figure 1. Signaling pathways and the corresponding miRs implicated in MetS- associated cancer. Several miRs are deregulated in both MetS/obesity and various types of cancers. Mir-21 is regulated by B-catenin via STAT3 and plays a role in oncogenesis and cancer progression through its direct targets TIMP3, which modulates matrix MMP activity and the PTEN/PI3K/AKT signaling pathway. PI3K/AKT represents the prevalent pathway implicated in carcinogenesis and MetS/obesity. The Mir221/222 cluster is implicated in both IR and cancer via CAV1, a key regulator for cell proliferation, apoptosis, migration, and metastasis. Other pathways implicated in cancer initiation and progression are: NOTCH-induced EMT, HMGA2, SOX2, c-Myc. TAM—tumor associated macrophages, MMP—matrix metalloproteinases, EMT—epithelial–mesenchymal transition, ECM–extracellular matrix, STAT3—signal transducer and activator of transcription 3, TIMP3—tissue inhibitor of metalloproteinase 3, PPAR-γ—peroxisome proliferator-activated receptor gamma, PTEN–phosphatase and tensin homolog, PI3K—phosphoinositide 3-kinase, AKT—protein kinase B, mTor—mammalian target of rapamycin, PPAR-α—Peroxisome proliferator activated receptor alpha, HMGA2—high mobility group AT-hook 2, SRC—Proto-oncogene tyrosine-protein kinase Src, SOX2—SRY-Box transcription factor 2, c-Myc—Cellular myelocytomatosis oncogene, CAV1—Caveolin 1.

2.1. The Role of miRNAs in Cancer by Modulating Macrophage Phenotypes

The inflammatory tumor microenvironment contains both innate immune cells (like, macrophages) and adaptive immune cells (B and T lymphocytes) [8]. Tumor-associated macrophages (TAMs) are derived mainly from circulating monocytes that are recruited into the tumor in response to various chemokines and growth factors produced by tumor and stromal cells.

Various signals, such as Toll-like receptor (TLR) ligands and interferon gamma (IFN-γ) promote polarization of TAMs to a M1-like proinflammatory phenotype, characterized by activation of proinflammatory genes, such as interleukin-1β and tumor necrosis factors (TNF)-α. Many factors promote polarization of TAMs to a M2-like phenotype (also termed alternatively activated), which express high levels of anti-inflammatory cytokines, scavenging receptors, angiogenic factors, and proteases that augment tumor progression by promoting angiogenesis, tumor cell invasion and metastasis, and suppress adaptive immunity [9,10]. Repolarization of TAMs to antitumor phenotypes is a potential therapeutic strategy of cancer [9].

MiRs may play a key role in tumorigenesis by promoting M2-like TAM polarization and inhibition of tumor infiltration with CD8+ cytotoxic T lymphocytes (CTLs). Deletion of the miRNA-processing enzyme DICER in macrophages stimulates M1-like TAM activation with recruitment of activated CTLs to the tumor. CTL-derived IFN-γ amplified M1 polarization of DICER1-deficient TAMs and inhibited tumor growth [11]. Overexpression of miR-511-3p in TAMs suppresses protumoral genes and inhibits tumor growth [12]. Increasing miR-511-3p activity in TAMs could be a potential therapeutic strategy to repolarize them to an antitumor phenotype. MiR-21-3p and -5p have an immunosuppressive effect by inhibiting the migration of CTLs into the tumor by decreasing the secretion of CCL-3 and CXCL-10 [13].

MiR-let-7b expression is upregulated in prostatic TAMs and promotes prostate carcinoma cell mobility and angiogenesis, while treatment with let-7b inhibitors reduce angiogenesis and cell mobility [14]. Several exosomal miRs have been shown to promote the M2-like phenotype of TAMs and accelerate tumor progression, such as miR-222-3p and miR-940 in epithelial ovarian cancer [15,16], miR-145 in colorectal cancer (CRC) cells [17], miR-103 in lung cancers [18]. Conversely, exosomal miR-21 could be transferred from M2-like TAM to gastric cancer cells, where it inhibits apoptosis of cancer cells through regulation of PI3K/Akt signaling and Bcl2 expression [19].

In contrast, miR-155 [20] and miR-19a-3p [21] expression is low in TAM and inhibits tumor growth and metastasis in a mouse breast cancer model by reprograming M2-like macrophages toward classic M1-like activation. Similarly, miR-142-3p is downregulated in TAM and inhibits glioma growth probably by promoting M2-like TAM apoptosis [22].

Cationic Bletilla Striata polysaccharide (cBSP) is a modified herb extract, which exhibits high affinity for macrophages and can be used as a nonviral drug delivery system targeting these cells [23]. The packaged let-7b into cBSP can be released in response to the tumor acidic microenvironment with the help of a pH-responsive material PEG-histamine-modified alginate [24]. This nanocomplex could reprogram TAMs towards M1-like and inhibit tumor growth in a breast cancer mouse model [24]. Otherwise, miR-18a was packed in a grapefruit-derived nanovector, which is specifically up taken by Kupffer cells, but not by other cells, after intravenous administration [25]. MiR-18a inhibits liver metastasis of colon cancer by inducing M1-like polarization. The therapeutic application of macrophage-derived miRNAs by using macrophage-specific delivery systems is a promising means.

2.2. The Role of CTBP1 in Cancer by Modulating microRNAs Expression

C-terminal binding protein 1 (CTBP1) is a co-repressor of many tumor suppressor genes that is activated by either NAD+ or NADH. However, affinity of CTBP1 for NADH is 100-fold higher, making it a molecular sensor of the metabolic state of the cell [26]. MetS-like disease, generated by chronic high fat diet (HFD), increases intracellular NADH and activates CTBP1. CTBP1 represses the gene expression of epithelial cell adhesion molecules, like E-cadherin, and promotes epithelial to mesenchymal transition (EMT), which contributes to cell proliferation and invasion [27]. CTBP1 upregulation and the resulting E-cadherin downregulation were correlated with the progression of human hepatocellular carcinoma (HCC). CTBP1 increased breast [28] and prostate [29] tumor growth and metastasis, in MetS mice, by modulating multiple genes and miRNAs expression implicated in the extracellular matrix (ECM), cell adhesion, and cell proliferation. Thus, CTBP1 regulates 42 miRNAs in MetS mice with breast cancer. Several miRNAs were previously reported altered in cancer, such as let-7e-3p, miR-4448, miR-223-3p, miR-3151-5p, miR-940, miR-378a-3p, miR-146a-5p, and miR-124 [28]. Some of them have been involved in breast cancer progression and metastasis, miR-378a-3p, miR-146a-5p, let-7e-3p, miR-381-5p, miR-194-5p, miR-494-3p) [30]. In prostate cancer, CTBP1 regulates a cluster of miRNAs that target cell adhesion. As prostate cancer progresses in the setting of MetS, CTBP1 increases, resulting in repression of miR-205-5p together with upregulation of oncomiRs, like: miR-19b-3p, miR-29c-3p, miR-30b-5p, miR-301a-3p, miR-454-3p [29].

Altogether, these results suggest that CTBP1 hyperactivation is critical for MetS effect on cancer progression and metastasis since CTBP1 depletion diminishes the detection of circulating tumor cells [30] and the number and size of metastasis [29].

2.3. The Role of Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) in Obesity and Cancer

PPARγ is a transcription factor highly expressed in adipose tissue with a central role in differentiation and function of mature adipocytes. PPARγ functions as a tumor suppressor, it promotes apoptosis and inhibits cancer cell proliferation, angiogenesis, and tumor microenvironment inflammation [31,32]. Diet-induced obesity and/or IR induce a decline in the expression of PPARγ, with potential relevance in obesity-related cancers. Epigenetic regulation of PPARγ may explain its down-regulation, and several microRNAs have been implicated [33]. MiR-27b, 130b, and 138 are upregulated in obesity. MiR-27b and 130b target directly PPARγ [34,35], while miR-138 indirectly inhibits the expression of PPARγ [36]. The upregulation of miR-27b, 130b, and 138, as well as PPARγ promoter hypermethylation in obese patients was responsible for PPARγ suppression and susceptibility to CRC [37]. Similarly, microRNA-130b up-regulation promotes lung cancer progression by suppression of PPARγ, which in turn activates BCL-2 and VEGF-A [38]. Beside obesity, miR-27b may be upregulated by human papillomaviruses HPV16 E7 in cervical cancer tissue, which suppresses the expression of PPARγ and increases the level of oncogenic pH regulator, Na+/H+ exchanger isoform 1 (NHE1) [39].

Classically, PPARγ exerts an antifibrotic effect by antagonizing TGF-β signaling. However, an additional mechanism has been proposed recently. PPARγ was identified as a major transcription factor which regulates a class of 8 miRNAs with antifibrotic properties, i.e., miR-29c, miR-335, miR-338, let-7a, let-7c, let-7g, miR-30d, and miR-30e. This miRs network proved to be active in three human fibrosis-associated carcinomas: HCC, breast, and lung carcinomas [40].

2.4. PI3K/AKT—Common Pathway in MetS and Cancer

A common mechanism which might explain the crosstalk between MetS and cancer is the PI3K/AKT pathway which represents a direct target of several miRs and is disturbed in both conditions. Chakraborty et al. systematized the influence of a plethora of miRs acting on insulin signaling pathway such as: miR-320, miR-383, miR-181b on IGF-1/IGF1R; miR-128a, miR-96, miR-126 on IRS; miR-29, miR-384-5p, miR-1 on PI3K; miR-143, miR-145, miR-29, miR-383, miR-33a/b miR-21 on AKT/PKB; and miR-133a/b, miR-223, miR-143 on GLUT4 [41]. Additionally, miR-221 binds to PI3K mRNA and inhibits glucose uptake in HepG2 cells [42]. On the other site, the PI3K/AKT pathway represents the direct target of mir-221 in several types of cancer, like laryngeal cancer [43], pancreatic cancer [44], breast cancer [45], and prostate cancer [46].

Striking evidence of a crosstalk between adipose tissue and prostate cancer, through miRNAs, has been recently reported by Massillo et al. [46]. In their study, mice with MetS characteristics, induced by a high-fat diet, were injected with prostate cancer cells and assessed for tumor growth and miRs expression. The authors found a group of 5 miRNAs (miR-221-3p, 27a-3p, 34a-5p, 138- 5p, and 146a- 5p) that were increased in gonadal adipose tissue, prostate tumors, and plasma of MetS mice compared to control animals. From these, miR-221-3p, 27a-3p, 34a-5p, and 146a-5p were confirmed to be important in prostate cancer patients [46].

MiR-221-3p proved again to mediate the crosstalk between adipose tissue and tumor growth in breast cancer [47]. The overexpression of mir-221-3p in human adipocytes impairs adipocyte lipid storage and differentiation, while conditioned medium obtained from miR-221-3p overexpressing adipocytes increased the invasion and proliferation of MCF-7 cells. Of great interest is the fact that the expression of mir-221-3p in subjects undergoing mastectomy, in the adipose tissue adjacent to BC, increases with the grade of BC. A negative correlation between the overexpression of mir-221-3p and AdipoQ was noticed, thus the inhibitory effects on BC growth of AdipoQ were lost [47].

Additionally, the PIK/AKT signaling pathway represents a target for mir-145. The upregulation of mir-145 in obese mice prevents insulin-stimulated AKT activation [41]. The overexpression of mir-145 in CRC leads to the inhibition of the PI3K/AKT signaling pathway, which in turn increases the sensitivity CRC to oxaliplatin [48]

A recent multi-omics approach and computational analysis on human visceral adipocytes compared the dysregulated miRNAs in obese subjects with or without CRC with normal weight controls [49]. MiR-193b-3p, miR- 125a-5p, and miR-1247-5p, were found to be downregulated in both cancer and obese conditions. Both miR-193b-3p and miR-1247-5p act as tumor suppressors in different types of cancer, suggesting that their repression in adipose tissue from obese and CRC individuals could have a potential tumorigenic role. Several pathways were dysregulated in both obesity and CRC networks: inflammatory signaling, such as IL-37 and IL-13, TGF-beta signaling, PTEN regulation, type I IFN signaling, SUMOylation, RNA metabolism, pathways related to vesicle budding and endocytosis [49].

2.5. Caveolin-1 (CAV1) Signaling

CAV1 is the main component of caveolae which are small invaginations at the plasma membrane, especially in endothelial cells and adipocytes. They are involved in endocytic and exocytic pathways as well as signal transduction.

CAV1 a critical regulator of the insulin receptor and insulin signaling, by stabilizing caveolae and their associated insulin receptors [50]. CAV1 is targeted by several miRs, which are upregulated in obesity and IR, like miR-103/107 [50] and miR-221/222 [51]. Upregulation of CAV-1 upon miR-103/107 or miR-221/222 inactivation improves insulin sensitivity and decreases glucose levels [50,51]. Interestingly, the expression on miR-221/222 correlated with BMI and HOMA-IR in postmenopausal women, with DM2 and/or breast cancer, but the highest serum levels were found in women with both diseases [51].

Downregulation of CAV1 in breast cancer promotes tumor relapse, drug resistance, and poor outcome [52,53], and has been related to the increased expression of several growth factors and regulators, like stromal cell-derived factor-1 (SDF-1), epidermal growth factor (EGF), and fibroblast-specific protein-1 (FSP-1) [54].

It has been shown that hyperglycemia induces epithelial to mesenchymal transition (EMT), a key process in metastatic disease, in MCF-7 and T47D human breast cancer cells. By blocking the hyperglycemia-induced EMT phenotype, cell growth was suppressed but invasive capacity increased through a CAV-1 dependent mechanism [55]. Similarly, diet-induced obesity increased melanoma progression in male C57BL/6J mice by enhancing Cav-1 and FASN expression in tumors [56].

2.6. Wnt/β-catenin Signaling

The canonical Wnt/β-cate pathway is involved both in cancer and in various metabolic processes including adipogenesis and glucose homeostasis [57]. Dysregulation of the Wnt/β-catenin pathway is one of the most relevant driving forces in cancer development and metastasis. Several miRs have been implicated in different types of cancer, like: CRC, breast cancer, ovarian, prostate cancer, through Wnt/β-catenin pathway dysregulation [58].

The mechanism of increased risk of HCC in obese individuals was recently elucidated [59]. It has been demonstrated that infiltrating macrophages induced by liver steatosis promotes growth of tumor progenitor cells through Wnt/β-catenin activation. Indeed, activation of Wnt signaling predicts 90% of tumors in a large cohort of patient samples [59]. Moreover, the obesity-related upregulation of miR-27a has been shown to promote metastasis of HCC through activated Wnt/β-catenin signaling [60].

2.7. PTEN Signaling

The tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid phosphatase with role in obesity and IR [61] and several cancers [62]. PTEN is a potent tumor suppressor, and its loss of function is frequently encountered in

cancer. Even a slight decrease in PTEN levels and activity results in cancer susceptibility or tumor progression.

Mostly through the activation of the PI3K/AKT/mTOR pathway, PTEN deficiency influences a variety of biologic processes that sustain cancer cell growth and proliferation [63]. Because metabolic effects of insulin are mediated through the same pathway, PTEN has a critical role in modulating insulin-induced glucose uptake and insulin-induced suppression of hepatic gluconeogenesis. Several miRs have been shown to inhibit the expression of PTEN at the post-transcriptional level in a variety of cancer types, like miR-21, miR-22, miR-214, mir-17–92, mir-106b-25, mir-367–302b, and mir-221–222 [64].

Interestingly, it has been shown that astrocyte-derived exosomes, containing anti-PTEN microRNAs, suppress PTEN expression in the brain metastatic tumor cells [65]. Recently, polyphenols proved to have chemopreventive potential in obesity-induced prostate cancer by rescuing PTEN expression [66].

PTEN interacts and facilitates TGF-β impacts on cell proliferation. Up regulation of PTEN diminishes TGF-β-mediated AKT phosphorylation, precluding the functions of TGFβ on cell proliferation while its down-regulation promotes TGF-β effects on induction of the PI3K pathway.

2.8. The miRNA-Processing Enzyme DICER

DICER has a central role in the final steps of the miRs processing pathway. It has been involved in both pancreas function and insulin signaling [67]. More recently, it has been shown that adipose tissue is an important source of circulating exosomal miRs, which can regulate gene expression in distant tissues. Adipose-tissue-specific knockout of DICER in mice, as well as humans with lipodystrophy, significantly decreased the circulating levels of exosomal miRs [68].

Upon deletion of DICER, several molecules and receptor tyrosine kinases, which are involved in IR and cancer development, were hyperphosphorylated (IGF1, IR, IRS-2, and STAT3) [69].

Many studies revealed that DICER acts as a tumor suppressor and loss of DICER protein expression in invasive tumor samples. Low DICER expression was associated with poor prognosis in ovarian cancer (HR = 1.93), otorhinolaryngological tumors (HR = 2.39), hematological cancers (HR = 2.45), and neuroblastoma (HR = 4.03) [70]. Several miRs have been shown to target and repress DICER, like: miR-103/miR-107 in breast cancer [71], miR-221/miR-222 and miR-29a in triple negative breast cancers [72]. Similarly, miR-103 target and suppressed DICER and PTEN, promoting proliferation and migration of CRC [73].

In contrast, transcriptional activation of DICER through ERK/Sp1 activation causes pancreatic cancer progression and resistance to gemcitabine [74].

2.9. PPARα -FOXP4- NOTCH Pathway

MiRs has been shown to regulate PPARα, a known regulator of both adipogenesis and carcinogenesis, suggesting that miRNAs play a vital role between obesity and cancer [75].

PPARα was demonstrated to act as an oncogene, especially in breast cancer. Thus, Chen et al. showed that ectopic expression of PPARα increases cell proliferation of breast and pancreatic cancer cells via a novel target gene, *carnitine palmitoyl transferase 1C* (*CPT1C*) [76]. Interestingly, coculture of breast cancer cells with mature adipocytes increased secretion of proinflammatory cytokines and chemokines and consequently the proliferation, migration, and invasive of cancer cells. The most deregulated miRs were miR-3184-5p (upregulated) and miR-181c-3p (downregulated) and they target directly FOXP4 and PPARα, respectively [77]. These data strongly proved that adipocytes-secreted factors drive the breast cancer progression and metastasis in obese patients. FOXP4 is a family of forkhead box transcription factors, with a critical role in cancer growth and metastasis in different types of cancer. Likewise, FOXP4 expression was negatively regulated by miR-3184-5p in NSCLC [78].

The Notch signaling pathway is a highly conserved pathway, which is required for cell–cell communication and many biological processes such as proliferation, organ development, differentiation, metabolism, and maintenance of stem cells [79]. The activation of the Notch pathway is associated with poor patient survival in breast cancer [80]. More interestingly, a recent study demonstrated that activation of Notch induces dedifferentiation of mature adipocytes and promotes tumorigenic transformation in mice [81]. Battle et al. [82] demonstrated the role of obesity-induced leptin-Notch signaling in breast cancer. These studies support the concept that adipocytes activate in the breast tumor microenvironment, the Notch-EMT signaling, increasing the migration and invasion, which in turn, promote a more aggressive metastatic tumor.

2.10. SRC/SOX2/c-MYC Pathway

It has been shown recently that co-culture of breast cancer cells with immature adipocytes or cytokines upregulates miR-302b via activation of SRC. Picon-Ruiz et al. [83] showed that the SRC action sustains cytokine induction and promotes SOX2-dependent miR-302b upregulation, to further induce MYC and SOX2 expression and increase malignant stem cells. Feed-forward mechanisms established by SRC-driven SOX2 and miR-302b induction would sustain subsequent cytokine production after initial exposures.

SRC was shown to activate NF-kB via STAT3 to induce IL6 and oppose Let7-mediated IL6 repression [84]. SOX2 is amplified in certain cancers and can drive clonogenic tumor growth [85]. Moreover, miR-302b expression in breast cancers was associated with expression of stem cell markers, nodal metastasis, and poor patient outcome [86].

MiR-21

MiR-21 levels were found to be increased in patients with type 1 (T1D) and type 2 diabetes (T2D), the circulating levels of miR-21 reflecting the degree of pancreatic inflammation [87]. Contrarily, there are studies that did not find discrepancies in the miR-21 serum levels in patients with or without diabetes [88]. Interestingly, miR-21 antagomir ameliorates metabolic disturbances in T2D patients by up-regulating the expression of the target gene *TIMP3* [89]. Furthermore, the inhibition of the miR-21 expression may represent a key point in the improvement of glycemic control through PPARc and GLUT4 regulation [90]. The expression of mir-21 is reduced in insulin-resistant adipocytes. Additionally, the over-expression of mir-21 improved glucose metabolism and insulinemia via the PTEN/PI3K/Akt pathway [91].

Low levels of miR-21 were identified in the peripheral blood mononuclear cells of obese, regardless of the presence of diabetes, which was negatively correlated with inflammatory cytokine production [92]. By targeting 3′UTR of STAT3 mRNA, miR-21 regulates the adipose cell proliferation and differentiation [93]. Additionally, the anti-miR-21 proved to be efficient for miR-21 inhibition which consequently led to weight loss via its target genes: transforming growth factor beta receptor 2 (TGFRB2), PTEN, and Sprouty1 and 2 [94]. In subjects with MetS, the circulatory levels of mir-21 were decreased compared to the patients without MetS [95].

Dysregulation in the expression miR-21 was also observed in neoplasms, miR-21 being one of the most studied oncomirs (see Table 1). Thus, its overexpression promotes metastatic phenotype of cancers by targeting RHOB and suppressing its activity [96].

MiR-21 is a determinant of prostate cancer aggressiveness by targeting 3′-UTRs of PDCD4 and maspin. Upregulation of mir-21 was mediated by NADPH oxidase system-ROS generation—Akt pathway [97]. Similarly, increased levels of mir-21 were associated with the prostate cancer aggressiveness, thus being useful in the identification of high-risk patients [98]. MiR-21 emerged as an independent risk factor for recurrence of prostate neoplasia in patients with obesity but not in non-obese subjects [99].

As mentioned above, miR-21 exerts its oncogenic effect on HHC cells by regulating the protein kinase B (AKT)/extracellular signal-regulated kinase (ERK) pathways [100].

As other miRs, mir-21 is overexpressed in osteosarcoma tissue playing a key role in tumor invasion and migration, having the tumor suppressor gene, RECK, as a target in the

aforementioned process [101]. MiR-21 implication in CRC was intensively studied. The interplay between CASC7, mir-21, and IGN3 might have a key role in CRC progression [102]. The long intergenic noncoding RNA, LINC00312 disrupts the proliferation, migration, and invasion of CRC cells by reducing the expression of miR-21 and consequently increasing PTEN expression [103]. MiR-21 promotes the development of CRC by targeting directly RHOB, thus promoting the cancer cells proliferation, invasion, and inhibiting the programmed cell death [104]. Additionally, miR-21 promotes the CRC through the downregulation of Sec23A [105]. The inhibition of miR-21 and the consequent increased expression of *TIMP*-3 and *RECK* proved to reduce the aggressiveness and the ability to metastasize [250].

The increased expression of oncomir-21 was identified in breast cancer, being correlated with circulating hormone levels. It plays an important role in tumor progression and aggressiveness by targeting STAT3 [106] and PI3KR1 [107].

The overexpression of miR-21 in non-small cell lung cancer (NSCLC) was associated with enhanced tumor aggressiveness and invasiveness through PTEN deregulation [108].

The implication of miR-21 in the pathogenesis of papillary thyroid carcinoma is mediated by the VHL/PI3K/AKT pathway, thus increasing the aggressiveness of this type of cancer. In addition, VHL may be a valuable tool in order to counteract the effects exerted by miR-21 [109].

Gastric cancer progression is promoted by the overexpression of miR-21 which regulates the expression of tumor suppressor genes *PTEN* and *PDCD4* and consequently increases the cancers' aggressiveness [110]. The inhibition of miR-21 in gastric cancer proved to be a promising strategy for counteracting its biological effects by reducing tumor aggression [251].

A potential mechanism that explains the aggressiveness of melanoma is the overexpression of oncomiR-21 that inhibits TIMP3 which regulates the matrix metalloproteinases activity. Therefore, the inhibition of miR-21 may be promising in melanoma management [111].

MiR-21/Sox2/β-catenin is one of the pathways implicated in glioma pathogenesis. Sox2 overexpression not only increased the expression of β-catenin but also enhanced tumor aggressiveness [112]. Additionally, miR-21 also plays a prognostic role in glioma patients [209]. The miR-21 is also correlated with the histological grade of gliomas and its expression is modulated by the STAT3/β-catenin pathway. Furthermore, the tumors' invasion capacities are augmented as a result of the regulatory effect of miR-21 over RECK [113].

MiR-21 is implicated in the pathogenesis of oral squamous cell carcinoma (OSCC) through its target gene, *PTEN*. Additionally, the expression of miR-21 and *PTEN* were associated with the tumors' grade [114]. Tongue squamous cell carcinoma oncomir-21 increased tumors' aggressiveness by inhibiting DKK and consequently activating the Wnt/β-Catenin pathway [115]. As in OSCC, the miR-21/PTEN pathway is implicated in cervical cancer pathogenesis [117].

The implication of miR-21 in the progression of renal cell cancer (RCC) was also established [118]. Regarding nasopharyngeal carcinoma, miR-21 expression proved to be up-regulated in advanced stages and was also correlated with the presence of metastatic adenopathy. Additionally, miR-21 seems to regulate the expression of BCL2 protein by targeting directly the BCL2 mRNA [116].

The aforementioned findings shed some light on the implication of miR-21 and its pathogenic pathways implicated in both MetS and cancer. Thus, miR-21 PTEN-induced deregulation is implicated in CRC [103], NSCLC [108], gastric cancer [110], OSCC [114], and cervical cancer [117], being closely correlated with the tumor aggressiveness [110], grade [114], and invasiveness [108]. Additionally, the miR-21/PTEN/PI3K/Akt pathway is implicated in the glucose metabolism and insulin homeostasis [91]. STAT3 represents a common target for miR-21 which is implicated in both breast cancer pathogenesis [106] and adipose cell proliferation and differentiation [93]. While the inhibition of miR-21 leads to an

increased expression of TIMP-3 and a consequent reduction in the CRC aggressiveness [250], the overexpression of miR-21 inhibits TIMP-3 in melanoma [111]. The up-regulation of TIMP-3 improves the metabolic imbalances in T2D subjects [89]. Thus, PTEN/PI3K/Akt, STAT3, and TIMP-3 represent major pathways that mediate the crosstalk between MetS and cancer.

MiR-24-3p

MiR-24-3p is down-regulated in T2D and MetS subjects. MiRs levels correlated with serum insulin and HbA1c levels in individuals with T2D or MetS, and with higher BMI, dyslipidemia, and family history [252]. T2D patients expressed low levels of miR-24-3p [253]. Obesity induces overexpression of miR-24-3p which in turn suppresses HDL uptake, lipid metabolism, and steroid hormone intake by inhibiting Scavenger Receptor B-1 (SRB1) [254].

MiR-24-3p functions as a tumor suppressor. Overexpression of miR-24-3p inhibits p27Kip1 [122] and Bim expression, therefore increasing growth and proliferation of breast cancer [123].

In CRC, miR-24-3p has a tumor suppressor function, down-regulation of miR-24-3p promotes CRC development and progression and plays a potential role in prognosis and therapy. Overexpression of miR-24-3p inhibited cell proliferation, migration, and invasion, indicating a key role for dysregulation of miR-24-3p in CRC tumorigenesis, and might have a therapeutic potential to suppress CRC progression [120].

MiR-26a

MiR-26a is involved in insulin signaling pathways through its action on GSK3β, PKCδ, and PKCθ, in fatty acid metabolism and gluconeogenesis through its effect on the genes that regulate PCK1 and TCF7L2 expression. It was demonstrated in animal models that a slight decrease in miR-26a contributes to the development of IR and T2D, and that a slight increase can prevent the development of complications associated with obesity [255]. At the same time, it was proved that miR-26a is a potent inhibitor of adipocyte differentiation, inhibiting adipogenesis through its suppressive action on Fxl19 [256].

MiR-26a plays a dual role in tumorigenesis, functioning both as a tumor suppressor and as an oncomir. Studies have shown that miR-26a acts as a tumor suppressor in lung cancer, breast cancer, HCC, rhabdomyosarcoma, prostate cancer, melanoma, papillary thyroid carcinoma, gastrointestinal carcinomas through its action on key proteins involved in the control of cell proliferation such as p53, SMAD1, EZH2, IL-6-Stat3, CTDSP1/2/L, SODD, CKS2, FGF9. On the other hand, miR-26a is involved in tumor pathogenesis through its oncogenic effect. For example, in glioma, miR-26a acts as a tumor promoter through its action on the PTEN and PHB signaling pathways, in ovarian cancer through effect on ERα, and in cholangiocarcinoma by reducing GSK3 [124].

MiR-26 was involved in triple-negative breast cancer development by down-regulating the expression of BRCA1 [126]. Additionally, in breast cancer, miR-26 expression was correlated with tumor size, HER2 status, and ki-67 value [125].

An interesting clinical implication could be the use of miR-26a for therapeutic purposes, a number of studies demonstrating that it can influence sensitivity to conventional chemotherapy [124]. For example, in pancreatic cancer, miR-26a overexpression facilitates local accumulation and sensitivity to gemcitabine [257].

Mir-26b

MiR-26b is among the obesity-related miRNAs, being reduced in adipocytes from visceral obesity and IR. The expression level of miR-26b negatively correlates with increasing BMI and IR in human obese subjects [258]. MiR-26b promotes Glut4 translocation to the plasma membrane and insulin-stimulated glucose uptake in human mature adipocytes. MiR-26b increases insulin sensitivity via the PTEN/PI3K/AKT pathway [127].

The MiR-26 family of microRNAs (miR-26a-1, miR-26a-2, and miR-26b) was proved to be a major inhibitor of adipogenesis, at least in part, by repressing expression of Fbxl19 [116]. Deletion of all miR-26 in mice resulted in excess body fat and dyslipidemia in animals fed normal chow due to precocious differentiation of adipocyte progenitor cells [126].

MiR-26b was shown to be down regulated and exhibited an antitumor effect in glioma and lung cancer cells by targeting cyclooxygenase (COX)-2 [127,128], and in HCC by targeting EphA2 [129]. Overexpression of miR-26b inhibited proliferation, invasion, and migration of cancer cells and might be a therapeutic strategy in these tumors [127,129].

MiR-27

MiR-27 has shown to be overexpressed in obesity due to hypoxic condition. The miR-27 family inhibits PPAR-γ function, activates Wnt1 signaling [259], and suppresses GLUT-4 and PI3K signaling, leading to hyperglycemia, IR, and hyperlipidemia [34].

The MiR-27 family was upregulated in multipotent stem cells isolated from omentum of morbidly obese patient compared to lean subjects, leading to a dysregulation of important pathways implicated in the early stages of adipocyte differentiation such as Wnt, TGFβ/Smad, and PPARγ/C/EBPα pathways [260]. Kang et al. described MiR-27 (miR-27a and miR-27b) as an anti-adipogenic microRNA by targeting prohibitin (PHB) and impairing mitochondrial function, which leads to a reduction of adipogenesis [131].

MiR-27 acts as a tumor suppressor in breast cancer, by targeting SPRY1, BAK, FOXO1, and CBLB/GRB2 [132,261,262]. Upregulation of miRNA-27 was correlated with a higher risk of gastric cancer, by promoting transformation of cancer-associated fibroblasts [130].

MiR-27b

MiR-27b was shown to be upregulated in different models of IR [131]. miR-27b directly suppresses the expression of insulin receptor (INSR) by targeting 3'UTR of INSR. Modulation of miR-27b expression in a HFD-induced IR mice model improved glucose tolerance and insulin sensitivity in adipose tissue by increasing the expression of its target gene INSR [131].

The upregulation of miR- 27b in obese patients was associated with susceptibility to CRC through PPARγ promoter hypermethylation and downregulation [37].

MiR-27b was upregulated in cervical cancer cells and tissues [263]. It acts as an oncogene with a role in the progression of cervical cancer. Upregulation of miR-27b increased proliferation, cell cycle transition from G1 to S phase, migration, and invasion of C33A cells, by modulating cadherin 11 (CDH11) and EMT [133]. Recently, it has been shown that miR-27b may be upregulated by human papillomaviruses HPV16 E7, which suppresses the expression of PPARγ and increases the level of Na+/H+ exchanger isoform 1 (NHE1) [39].

Contrary, MiR-27b has been reported as a tumor suppressor in several cancers. MiR-27b was decreased in both NSCLC tissues and cell lines, while its overexpression suppressed NSCLC cells proliferation and invasion, by targeting LIM kinase 1 (LIMK1) [264] and Sp1 [134].

Mir-30

The miRs expression analysis in ATMs of high fat diet (HFD)-induced obesity in mice compared to lean normal chow diet mice revealed substantial dysregulations of miR-30 which led to a M1 polarization of ATM in the HFD mice. The inhibition of miR-30 proved to induce inflammation through the DLL4-Notch signaling-pathway, thus the anti-inflammatory role of miR-30 in macrophages [265].

Aside from obesity and inflammation, miR-30 is also implicated in breast cancer [135], NSCLC [136], and pancreatic cancer [137]. In breast cancer cells, miR-30 was the highest expressed miRs and its expression was much higher in invasive tumor cells than in mass tumor cells. Additionally, silencing miR-30 reduced the invasion and growth abilities of cancer cells but at the same time prolonged their survival time [135].

In NSCLC, the levels of miR-30 are down-regulated which leads to the overexpression of MMP19 in pulmonary cancer. MMP19 is implicated in the promotion of tumor genesis and metastasis so it can be a predictor of a poor prognosis in lung cancer patients. Explanatory for the prognostic implications of miR-30/MMP19 pathway is the effect of MMP19 on EMT that results in loss of intercellular adhesion. Furthermore, MMP19 is responsible for high expression levels of proliferative growth factors [136].

In pancreatic cancer, miR-30 was implicated in gemcitabine treatment resistant cancer lines and increased invasiveness but had no impact on cell proliferation [137].

MiR-31

MiR-31 has a higher expression in visceral adipose tissue of obese and diabetic patients compared to healthy subjects, but it is not clear yet if this is the cause or the consequence of obesity or T2D. The target genes are involved in adipogenesis (*PPARg*, *PRKAA1*, and *ACACA*) and insulin signaling (*GLUT4* and *IRS1*) [266]. The secretion of miR-31 from adipose tissue-derived stem cells promote angiogenesis by targeting the factor inhibiting HIF-1a, indicating a possible correlation between dysfunction of adipose tissue and tumorigenesis [267]. The expression of miR-31 in tumor tissue of patients with head and neck squamous cell carcinoma (HNSCC) was higher than that in adjacent normal tissues. Highly expressed MiR-31 was associated with tumor differentiation, metastasis, and poor prognosis. The expression of miR-31 significantly reduced the expression of the tumor suppressor gene, *adenine thymine-rich interactive domain 1A* (*ARIDIA*) [138]. Similarly, higher miR-31 expression was detected in rectal cancer tissues compared with adjacent tissues [139]. In vitro overexpression of miR-31 increases invasiveness, while the proliferation and invasion of rectal cancer cells were inhibited by inhibiting the expression of miR- 31 [139]. Similarly, increased miR-31 expression in CRC tissue was associated with disease invasiveness and poor prognosis, by targeting factor-inhibiting HIF-1α (FIH-1) [140].

MiR-31 proved to have a dual effect on breast cancer. It increases the primary tumor growth but most important, it is key anti-metastatic miRs in breast cancer. MiR-31 expression was four-fold reduced in a non-metastatic breast cancer cell line (MCF-7), while its expression in a metastatic breast cancer line (MDA-MB231) was decreased 100-fold [141]. MiR-31 targets RhoA mRNA, which is implicated in cell movement and cytoskeleton. Another study confirmed that miR-31 expression is lost during breast cancer progression [268]. MiR-31 directly binds to the 3′-UTR of G protein alpha-13 (GNA13) and suppresses its activity. GNA13 is most highly expressed in more aggressive breast cancer cells [268].

Similarly, the pro-tumorigenic effect of miR-31 loss has been described in malignant mesothelioma (MM) of pleura [142]. MM cell lines derived from patients with aggressive tumors did not express miR-31 due to homozygous deletion of the miR-31-encoding gene that resides in 9p21.3. Re-introduction of miR-31 inhibited proliferation, migration, invasion, and clonogenicity of MM cells. The pro-survival phosphatase PPP6C possesses three potential binding sites for miR-31 in its 3′-UTR and was down-regulated by miR-31 introduction and up-regulated in clinical MM specimens [142].

MiR-31 demonstrated a tumor suppressive role in glioma tumor. Its expression was markedly reduced both in glioma cell lines and in glioma tumor specimens compared with the adjacent human brain tissues [143]. miR-31 downregulation was due to hypermethylation of its promoter region. It modulates Dock1 expression, while Dock1 promotes the IL8-induced chemotaxis and mesenchymal transition of glioma cells through the NF-jB/Snail signaling pathway. Moreover, the therapy with a DNA methyltransferase inhibitor restored miR-31 expression in glioma cells and inhibited cell invasion. Similarly, miR-31 is downregulated and acts as a tumor suppressor in gastric [269] and serous ovarian cancer cell lines as well in serous ovarian tumors via regulation of zeste homolog 2 (ZH2) [144]. Overexpression of miR-31 causes cell cycle arrest, inhibition of proliferation, migration, and invasion of the gastric, ovarian, osteosarcoma, and prostate cancer cell lines [144,269].

MiR-34a

Overexpression of miR-34a in visceral fat of overweight/obese subjects is associated with IR and metabolic inflammation. Lipid loaded mature adipocyte-secreted exosomes transport miR-34a to macrophages and suppress the anti-inflammatory M2 phenotype by repressing Kruppel-like factor 4 (Klf-4) [270]. MiR-34a is a key mediator in exacerbating obesity-related systemic inflammation and metabolic dysregulation [270]. MiR-34 was also described to be increased in humans with non-alcoholic fatty liver disease (NAFLD) and T2D [271,272], and some experimental evidence indicates that increased miR-34a levels in the liver are associated with metabolic alterations [273]. MiR-34 suppresses WAT

browning in obesity, by targeting Fibroblast Growth Factor Receptor 19 (FGFR19) and SIRT1 (sirtuin 1). SIRT1 suppression is linked to impaired transcriptional regulation of brown and beige markers through deacetylation of PPARGC1-α [274].

Many studies reported that miR-34a is down-regulated and acts as a tumor suppressor in human breast tissue [157]. MiR-34a acts as a tumor suppressor miR by down-regulating its target genes such as *BCL*-2 and *SIRT1* [145] and *Notch1* [146], Wnt/β-catenin signaling pathway [147], *fra*-1 [148] and *MYC* [149].

In CRC, Siemens et al. showed that the formation of distant metastases is associated with epigenetic silencing of miR-34a in primary tumors. In addition, they found that patients who subsequently developed distant metastases had a preferential up-regulation of the miR-34a targets *Snail*, c-*Met*, and β-*catenin*, which have prometastatic functions, in the primary tumors. Notably, the authors indicate that the detection of enhanced c-Met and β-catenin expression alongside miR-34a CpG methylation can have prognostic value [152].

Fujita et al. studied the role of miR-34a in anticancer drug resistance in prostate cancer cell lines. They found a significantly reduced expression of miR-34a in p53-null PC3 cells and p53-mutated DU145 cells compared to wild-type p53 LNCaP cells. Ectopic expression of miR-34a can decrease SIRT1 mRNA and protein levels, lead to cell cycle arrest and growth inhibition, and mitigate chemoresistance to the anticancer drug camptothecin by inducing apoptosis [275].

MiR-96

MiR-96 targets directly the 3'UTRs of INSR and IRS-1, and decreases their expression at the post-transcriptional level. Induction of miR-96 by dietary saturated fatty acids impairs insulin signaling and exacerbates hepatic insulin resistance through the suppression of INSR and IRS-1 [276]. Grape seed proanthocyanidins extracts significantly decreases HFD-induced miR-96 upregulation in mice, and reduces the expressions of miR-96 downstream molecules, FOXO1, p-mTOR, mTOR, and LC3A/B [277].

MiR-96 functions as an oncogene in several types of cancers. Thus, miR-96 is overexpressed in HCC and promotes cell proliferation, migration, and invasion by inhibiting the tumor suppressors SOX6 [159] and EphrinA5 [160]. It is upregulated in breast cancer and enhances tumor growth and progression by silencing the protein tyrosine phosphatase, *PTPN9* [161] and the tumor suppressor genes *FOXO1* [162] and *FOXO3a* [163]. PTPN9 may contribute to tumor suppression by dephosphorylation and silencing of EGFR, ErbB2, and STAT3 in breast cancer [161]. Similarly, miR-96 is significantly upregulated in NSCLC and functions as an onco-miRNA via targeting FOXO3 [164]. MiR-96 up-regulation was demonstrated in esophageal cancer, where it promotes proliferation and chemo- or radio resistance by RECK down-regulation [165].

Contrary, miR-96 may act as a tumor suppressor, inhibiting migration, invasion, and proliferation of glioblastoma multiforme cells via down regulation of astrocyte elevated gene-1 (AEG-1) at the mRNA and protein levels [166]. It suppresses renal cell carcinoma invasion by downregulation of Ezrin expression [167]. Similarly, Yu et al. showed that miR-96 functions as a tumor-suppressor in pancreatic cancer cells, where it decreased cell migration and invasion and decreased tumor growth via downregulation of *KRAS* oncogene [168]. More recently, it has been shown that lncRNA TP53TG1 is upregulated in pancreatic ductal adenocarcinoma and contributes to the tumor growth and progression. TP53TG1 operates as a sponge for miR-96 to weaken the suppressive effect of miR-96 on KRAS, and thus increases the expression of KRAS [278].

MiR-100

It has been demonstrated that normoglycemic and T2D obese patients have a reduced miR-100 expression. Obese patients with T2D show a much more reduced expression compared to normoglycemic obese patients, particularly in visceral adipose tissue compared to subcutaneous tissue. These low values are correlated with high values of TGL, basal glycemia and hsCRP. Low miR-100 induces an increase in VLDLR and differentiation of preadipocytes into mature adipocytes capable of accumulating higher lipid amounts, thus contributing to the pathogenesis of obesity.

The mTOR and IGFR signaling pathways represent direct targets of miR-100, being involved in adipogenesis, IR, as well as carcinogenesis. Inhibition of mTOR by rapamycin inhibits adipogenesis through its effect on PPAR-γ activity. IGFR mediates similar metabolic effects regarding glucose influx and IR adipogenesis. Because of structural similarities, IGFR and IR can form hybrid IR receptors [279].

Regarding carcinogenesis, miR-100 overexpression inhibits the transcription of a number of proteins in the IGF/mTOR signaling pathway, such as IGF1R, IGF2, MCL1, with a role in tumor proliferation and survival and involved in the development of several types of cancers: urinary bladder urothelial carcinoma, chondrosarcoma, endometrioid endometrial carcinoma, breast carcinoma, esophageal squamous cell carcinoma (ESCC), acute leukemia, pancreatic adenocarcinoma, H&N cancers, HCC, prostatic adenocarcinoma, NSCLC [280].

In addition to its pathogenic role, miR-100 also has a potential diagnostic, prognostic, and therapeutic role. For example, in urinary bladder cancer, studies have demonstrated that low miR-100 values represent an independent negative prognostic factor that is correlated with shorter PFS (progression-free survival) and OS (overall survival), and might be a useful instrument in patient stratification [169]. Similarly, the negative prognostic role of low miR-100 expression was demonstrated for HCC, RCC, bladder cancer, NSCLC and epithelial ovarian cancer [170], endometrioid endometrial carcinoma [171], CRC [172].

MiR-125b

A study showed that hepatic miR-125b expression is much higher in persons with T2DM compared to healthy individuals, and this contributes to the development of insulin resistance. The inhibition of endogenous miR-125b contributes to increasing insulin sensitivity in insulin resistance conditions. The authors also demonstrated that this effect is the result of the action of miR-125b on PI3K, which determines a decrease of insulin-induced AKT phosphorylation in hepatocytes [281].

In contrast, a study on animals showed that miR-125b expression is lower in the pancreatic cells of mice with T2D, but these presented a higher expression of DACT1, JNK, and c-Jun, demonstrating that miR-125b stimulates the increase of insulin sensitivity and pancreatic beta cell function through the inhibition of the JNK signaling pathway due to the suppressive effect on DACT1 [282].

SIRTs represent a group of enzymes with an important role in cell metabolism, inflammation, reactive oxygen species (ROS) production, and in the balance between apoptosis, survival, and cell proliferation. It was demonstrated that SIRT1 values are lower in the adipose tissue of obese persons compared to normal weight subjects, and that they are negatively correlated with miR-125b expression. SIRT1 stimulates lipolysis through its action on FOXO1 and suppresses the expression of some pro-inflammatory genes in the adipocytes and macrophages present in adipose tissue by interference with the NF-κB signaling pathway, which increases insulin sensitivity [283].

In cancer, miR-125b has an oncogenic effect in hematological cancers and a tumor suppressive effect in solid cancers. For example, in diffuse large B-cell lymphoma (DLBCL), constitutive activation of the NF-κB signaling pathway occurs through the suppressive effect of TNFAIP3 which normally inhibits the activation of this pathway by miR-125b [173]. Other targets of miR-125 involved in the development of hematological cancers are MAPK11, IRF4, and the TET2-VEGFA pathway in acute leukemia [174]. Tumor suppressive effects were observed in HCC, CRC, RCC, salivary gland carcinoma, thyroid cancer, laryngeal carcinoma, osteosarcoma, prostatic adenocarcinoma, melanoma, Ewing sarcoma, glioblastoma, gallbladder cancer through its action on mRNA genes, with effects on many pathways involved in carcinogenesis such as Wnt, PI3K/Akt, STAT-3, MAPK, NF-κB, p53 [175].

Overexpression of miR-125b was associated with resistance to cetuximab treatment in CRC and SCCHN, while in other cancers such as NSCLC, HCC, breast cancer, uterine cervical cancer, its overexpression increased sensitivity to chemotherapy. MiR-125b was also proposed as a potential marker of response to immunotherapy in NSCLC [284].

Mir-125b is implicated in the crosstalk between IR/MetS and cancer via its direct action on the PI3K/Akt pathway. Mir-125b decreases insulin sensitivity and disrupts glucose homeostasis by targeting PI3K in liver cells [281]. Additionally, mir-125 and its direct targets ErbB2, ErbB3 are implicated in prostate cancer initiation and progression through the PI3K/Akt pathway [285]

MiR-126

One of the targets of miR-126 is IRS-1, a protein with an important role in signal transduction in the insulin signaling pathway. It was demonstrated that low miR-126 expression is involved in the development of IR through the increase in the expression of these proteins [41]. Low values of miR-126 are also found in subjects with prediabetes compared to healthy individuals, a study even demonstrating the correlation of its serum values with the risk for subsequent T2D [286]. Another mechanism by which miR-126 is involved in the pathogenesis of inflammation and IR is through its effect on CCL-2 release from human adipocytes and macrophages [287].

In addition to the regulatory effect on the insulin signaling pathway [41], the miR-126/IRS-1 axis is involved in cancer pathogenesis [176]. IRS-1 disruptive effect on cell growth and DNA repair and replication might explain the mir-126 implication in cancer development and progression. MiR-126 acts as a tumor suppressor and a low expression has been observed in many cancers–carcinomas of the GI tract, lung, breast, prostate, thyroid. Downregulation of this miR facilitates tumor progression, migration, angiogenesis, and survival through its action on several genes and molecular pathways involved in oncogenesis such as *PI3K, KRAS, EGFL7, CRK, ADAM9, HOXA9, IRS-1, SOX-2, CADM1, PAX4, SLC7A5,* and *VEGF* [176].

Mir-130

Among white adipose tissue deregulated miRs, of great importance is miR-130 which might be overexpressed in the context of inflammatory stimulation, by TNF-alpha, leading to adipose cell dysfunction [177]. In diet induced obesity, mir-130 inhibition of APCDD1 leads to defective adipose cell differentiation through a plethora of laborious pathways [288]. Adolescents suffering from obesity proved to have higher plasmatic levels of mir-130 [289].

In gastric cancer cells, mir-130 enhanced their proliferation and invasion abilities. By targeting miR-130 with MRPL39, the aforementioned effects were counteracted through an anti-tumor effect [290].

Mir-143

Upregulation of mir-143 in dietary mouse models of obesity impairs insulin-stimulated AKT activation, through downregulation of oxysterol-binding protein-related protein 8 (ORP8) underlying the mechanism implicated in the obesity associated-IR [291]. Mir-143 expression proved to be deregulated in the mesenteric fat tissue of mice with high-fat-diet induced obesity. The overexpression of mir-143 was associated with leptin levels and IR. Furthermore, mir-143 disrupted the expression of PPARg and Ap2, adipocyte differentiation markers [292]. The overexpression of mir-143 was also identified in obese and morbidly obese patients [289,293]. Furthermore, the expression of mir-143 proved to be deregulated in the pediatric population suffering from obesity and its low levels were associated with disturbances in the lipid metabolism [289]. On the contrary, in the study conducted by Viesti A Collares R, no difference was identified regarding mir-143 expression between obese and non-obese patients [294].

While the overexpression of mir-143 is closely associated with IR [292] and obesity [291], its implication in oncogenesis was also established firstly through the inhibitory effect over the expression of *Bcl2, extracellular signal-regulated kinase-5(ERK5), and KRAS* [178]. Mir-143 proved to have antitumor effects in BC cells, thus abolishing the cancer cells growth by reducing the expression of *ERBB3* [179], *Kras, Vimentin, CXCR4, MMP-9* and increasing the expression of E-*Cadherin* [180]. Moreover, it acts synergically with miR-145, thus the cluster miR-143/145 exerts a greater anti-tumor effect than each individual miR [179].

The expression of miR-143 was also downregulated in ESCC. Mir-143 exerts its tumor suppressor capacities by targeting FAM83F and by inhibiting its activity, thus possessing anti-proliferative affects in ESCC cells [181]. Furthermore, miR-143 proved an anticancerous effect in gastric cancer (via DNMT3A) [182]. It has been shown that miR-143 has different expression patterns in colon vs. rectal cancer although the clinical implication of this phenomenon is unknown [295]. In the case of hepatocellular carcinoma, miR-143 regulates a plethora of genes. Thus, miR-143 downregulates the expression of *TLR2, NF-κB, MMP-2, MMP-9, CD44, MMP14, integrin β1,* and *integrin β4* and upregulates the expression of E-*cadherin* [183].

Regarding osteosarcoma, miR-143 reduces the tumors' capacity for lung metastasis with no influence on the neoplastic cell proliferation rate, mainly through the downregulation of *PAI*-1 [184].

TGF-B upregulates the miR-143 expression in NSCLC. Additionally, miR-143 suppressed the migration and invasion of NSCLC cells [185].

Taking into consideration the aforementioned findings, it is obvious that KRAS represents a crucial target that links the tumor suppressor miR-143 and various types of cancers (cervical cancer, prostate cancer, CRC, breast cancer NSCLC). KRAS is implicated in a plethora of essential pathways implicated in cancer cells proliferation, angiogenesis, invasion, and dissemination [296].

MiR-145

MiR-145 regulates AKT enzyme expression, which couples PI3K and GLUT4 activation, an important part in the insulin signaling pathway. A study demonstrated that obese mice present upregulation of miR-145, which prevents insulin-stimulated AKT activation [41]. Kirby et al. subsequently demonstrated in a study evaluating the expression of some miRs in subcutaneous adipose tissue in individuals with preserved insulin sensitivity and in persons with insulin resistance that miR-145 expression is at least three times lower in insulin resistant persons. In the same study, the authors evidence the target genes of miR-145: *ADAM22, MYO5A, LOX,* and *GM2A* [297].

Studies have shown that miR-145, along with miR-143, plays a role in controlling vascular homeostasis by regulating smooth muscle cell plasticity and responsiveness to the action of ACE (angiotensin-converting enzyme), which suggests a possible connection between the imbalance of miR-145/143 associated with obesity, increased cardiovascular risk, and poor blood pressure control, representing in this way a potential therapeutic target. It should be mentioned that low miR-143/145 expression was observed in several types of cancer, which might limit therapeutic potential in metabolic diseases [291].

MiR-145 is one of the most studied miRs in cancer, being involved in tumor proliferation, differentiation, apoptosis, metastasis, angiogenesis processes, as well as in therapeutic resistance. A meta-analysis showed that low miR-145 expression is associated with shorter OS in ovarian, CRC, glioma, osteosarcoma [186].

Like in the case of other miRs, the actions of miR-145 are multiple. In urinary bladder urothelial carcinoma, it has a suppressive action by acting on *KLF4*, which disturbs the Warburg effect and induces cell proliferation inhibition. Suppressive action has also been demonstrated in ESCC through the action on c-*Myc*, and in gastric cancer where miR-145 suppression increases *Ets1* expression, facilitating in this way tumor metastasis and angiogenesis. In TNBC, it plays a role in cell adhesion, regulating the activity of E-cadherin. Certain targets of miR-145 are involved in the pathogenesis of some cancers. Through the effect on the *FSCN1* gene, it is involved in the tumorigenesis of urinary bladder, esophageal, nasopharyngeal, liver, prostate cancer [186].

It has been demonstrated that miR-145 overexpression can increase the efficacy of chemotherapy. For example, in BC, miR-145 may induce intracellular doxorubicin accumulation through the suppressive effect on MRP1. The inhibition of the PI3K/AKT signaling pathway, which in turn induces MRP1 and P-gp inhibition, increases the sensitivity of esophageal squamous cell carcinoma to cisplatin and that of CRC to oxaliplatin. An increase

in tumor cell sensitivity to the cytotoxic action of cetuximab in CRC was also observed, through a reduction of BCL2 and an increase in the activity of caspases 3/7 [48].

Noteworthy is the implication of mir-145 in the development of breast cancer associated with T2D [298]. The mir-145/PI3K/Akt axis might represent a common pathway that links mir-145 expression to both MetS and cancer, taking into consideration its implication in the insulin signaling pathway [41] and in cancer pathogenesis [48].

MIR-155

MiR-155 is implicated in the glucose metabolism via C/EBPβand HDAC4. Overexpression of miR-155 was associated with an improvement of glucose serum levels. Additionally, the miR-155 knockout not only causes hyperglycemia but also increases the IR [299]. Upregulated miR-155 levels were also identified in the IR associated with Polycystic Ovary Syndrome (PCOS) model in rats. The IL-6/pSTAT3/miR-155/miR-21/PPAR-c pathway might represent the core mechanism which underlies the PCOS-associated IR [90].

MiR-155 has been found to be implicated in diet-induced obesity. Thus, miR-155 loss in mice resulted in less weight gain associated with a high-fat diet due to the upregulation of several genes implicated in adipogenesis (*Creb1, Cebpb, Pnpla2, Fabp4, Fasn, Ucp1, Cidea, PPARg*), insulin sensitivity (*Irs1, Glut4*), and inflammation (*AdipoQ*) [300]. A higher expression of miR-155, induced by NF-kB/TNF-alpha, was detected in subjects with obesity. Concerning the chronic inflammatory state associated with obesity, miR-155 mediates this process by inducing the expression of a plethora of genes and chemokines [301]. The adipose tissue associated macrophages represent a cellular source of miR-155 in the fat tissue [302]. In obese mice, ATM expressed higher levels of intracellular mir-155. The higher expression of miR-155 led to deregulations in the glucose metabolism, decreasing the glucose cellular uptake [7]. An interesting finding is that miR-155 may play a role in the mechanisms underlaying the obesity paradox [303].

Table 1. Adiposity-related miRs with potential role in cancer.

miRNAs	Target Genes and Functions in Obesity/IR/MeS	Target Genes or Pathway in Cancer	Type of Cancer (Oncomir/Tumor Suppressor)	References for Cancer Genes or Pathway
miR-21	TIMP3 PPAR-c, GLUT4 PTEN/PI3K/Akt pathway; STAT3, TGFRB2, PTEN, Sprouty1 and Sprouty 2	RhoB	HCC, Breast cancer ↑	[96]
		PDCD4 and Maspin	Prostate cancer ↑	[97,98]
			Prostate cancer ↓	[99]
		AKT/ERK pathways	HCC ↑	[100]
		RECK	Osteosarcoma ↑	[101]
		CASC7 and IGN3	Colorectal cancer ↑	[102]
		PTEN	Colorectal cancer ↑	[103]
		RhoB	Colorectal cancer ↑	[104]
		Sec23A	Colorectal cancer ↑	[105]
		TIMP-3 and RECK	Colorectal cancer ↑	[105]
		STAT3, PIK3R1	Breast cancer ↑	[106,107]
		PTEN	NSCLC ↑	[108]
		VHL/PI3K/AKT	Papillary thyroid cancer ↑	[109]
		PTEN and PDCD4	Gastric cancer ↑	[110]
		TIMP3	Melanoma ↑	[111]
		Sox2/β-catenin, RECK	Glioma ↑	[112,113]
		PTEN, DKK, BCL2	OSCC ↑	[114–116]
		PTEN	Cervical cancer ↑	[117]
			RCC ↑	[118]

Table 1. *Cont.*

miRNAs	Target Genes and Functions in Obesity/IR/MeS	Target Genes or Pathway in Cancer	Type of Cancer (Oncomir/Tumor Suppressor)	References for Cancer Genes or Pathway
miR-24-3p	SR-B1, HMGCR, DHCR24, SREBP2 KCNQ1	LPAATβ	Osteosarcoma ↓	[119]
		CCK8	Colorectal cancer ↓	[120,121]
		p27Kip1, Bim	Breast cancer ↓	[122,123]
miR-26a	GSK3β, PKCδ, PKCθ, ACSL3, ACSL4, PCK1, TCF7L2, FXl19	p53, SMAD1, EZH2, IL-6-Stat3, CTDSP1/2/L, SODD, CKS2, FGF9	Lung, breast, HCC, rabdomyosarcoma, prostate, melanoma, papillary thyroid, gastric, pancreatic cancer ↓	[124]
		MCL-1 BRCA1	Breast cancer ↓ Triple-negative BC↓	[125] [126]
		PTEN and PHB, ERα, GSK3	Glioma, ovarian cancer, colon cancer, cholangiocarcinoma ↑	[124]
miR-26b	Glut 4, PTEN/PI3K/AK, Fbxl19	Cox-2	Glioma, NSCLC ↓	[127,128]
		EphA2	HCC ↓	[129]
miR-27	PPAR-γ, Wnt1, GLUT-4 PI3K, PRDM16, PPARα, CREB, PGC1β	ZEB1, ZEB2, Slug, Vimentin, E-cadherin	Gastric cancer ↓	[130]
		MDR1/P-glycoprotein	Cancer cells ↑	[130]
		SPRY1, BAK, FOXO1, CBLB/GRB2	Breast cancer ↓	[131,132]
miR-27b	PHB, INSR, PPARγ	PPARγ	CRC ↑	[37]
		CDH11, EMT, PPARγ- NHE1 pathway	Cervical cancer ↑	[39,133]
		LIMK1, Sp1	NSCLC ↓	[48,134]
miR-30	DDL40-Notch-1		Breast cancer ↑	[135]
		MMP19	NSCLC ↑	[136]
		Fibronectin, Vimentin, N-cadherin	Pancreatic cancer ↑	[137]
miR-31	PPARg, PRKAA1, ACACA, GLUT4, IRS1, HIF-1a	ARIDIA	HNSCC ↑	[138]
			Rectal cancer ↑	[139]
		FIH-1	CRC ↑	[140]
		RhoA, GNA13	Breast cancer ↑	[140,141]
		PPP6C	Mezothelioma ↓	[142]
		Dock1, NF-jB/Snail	Glioma ↓	[143]
		ZH2, p53 pathway	Gastric, ovarian, osteosarcoma, prostate cancer ↓	[63,144]

Table 1. *Cont.*

miRNAs	Target Genes and Functions in Obesity/IR/MeS	Target Genes or Pathway in Cancer	Type of Cancer (Oncomir/Tumor Suppressor)	References for Cancer Genes or Pathway
miR-34a	Inhibit macrophage M2 induced adipose inflammation	BCL-2 and SIRT1	Breast cancer↓	[145]
		Notch1	Breast cancer↓	[146]
		Wnt/β-catenin signaling pathway	Breast cancer↓	[147]
		Fra-1	Breast cancer↓	[148]
		MYC, P53	Breast cancer↓	[149,150]
		E2F3	Neuroblastoma ↓	[151]
		c-Met and β-catenin	Colon cancer ↓	[152]
		P53	Osteosarcoma ↓	[153]
		MET, P53	Ovarian cancer ↓	[154,155]
		CD44	Prostatic cancer ↓	[156]
		AXL	Solid cancer ↓	[157,158]
miR96	INSR, IRS	SOX6, EphrinA5	HCC ↑	[159,160]
		PTPN9, FOXO1, FOXO3a	Breast cancer ↑	[161–163]
		FOXO3	NSCLC ↑	[164]
		RECK	ESCC ↑	[165]
		AEG-1	Glioblastoma ↓	[166]
		Ezrin	RCC ↓	[167]
		KRAS	Pancreatic cancer ↓	[168]
miR-100	mTOR, IGFR, VLDLR		Bladder cancer ↓	[169]
		HOXA1, Rac1, ICMT, EphB6, AGO2, Plk1, Wnt, β-catenin or RBSP3	HCC, RCC, bladder cancer, NSCLC, and epithelial ovarian cancer ↓	[170] [171]
		mTOR kinase	Endometrioid endometrial carcinoma ↓	[171]
			CRC ↓	[172]
miR-125b	PI3K/AKT JNK signaling pathway, SIRTs	NF-κB,	DLBCL ↑	[173]
		MAPK11, IRF4, TET2-VEGFA	Acute leukemia ↑	[174]
		Wnt, PI3K/Akt, STAT-3, MAPK, NF-κB, p53	HCC, CRC, RCC, thyroid larynx, osteosarcoma, prostate melanoma, Ewing sarcoma, glioblastoma, gallbladder, ovarian cancer ↓	[175]
miR-126	IRS-1, CCL2	PI3K, KRAS, EGFL7, CRK, ADAM9, HOXA9, IRS-1, SOX-2, SLC7A5 and VEGF	Gastrointestinal tract, genital tracts, breast, thyroid, lung cancers ↓	[176]
			NSCLC ↓	[177]
			CRC ↓	[176]
			RCC ↓	[176]

Table 1. *Cont.*

miRNAs	Target Genes and Functions in Obesity/IR/MeS	Target Genes or Pathway in Cancer	Type of Cancer (Oncomir/Tumor Suppressor)	References for Cancer Genes or Pathway
miR-143	ORP8/insulin-AKT pathway, PPARg and aP2 Leptin	Bcl2, ERK5, KRAS	Cervical, prostate, CRC ↓	[178]
		ERBB3	Breast cancer ↓	[179]
		KRAS, Vimentin, CXCR4, MMP-9	Breast cancer ↓	[180]
		FAM83F	Esophageal squamous cell carcinoma ↓	[181]
		DNMT3A	Gastric cancer ↓	[182]
		TLR2, NF-κB, MMP-2, MMP-9, CD44, MMP14, integrin β1, integrin β4	HCC ↓	[183]
		PAI-1/MMP-13	Osteosarcoma ↓	[184]
		Smad3, CD44, and K-Ras	NSCLC ↓	[185]
miR-145	AKT/PI3K/GLUT4 ADAM22, MYO5A, LOX, and GM2A	PI3K/AKT, MRP1, SMAD, KLF4, c-myc, Ets1, E-cadherin, FSCN1, BCL2	BC, gastric, CRC, NSCLC, glioma, HCC, osteosarcoma, ovarian, cervical, prostate, bladder, nasopharyngeal cancer ↓	[186]
miR-148a-3p	inhibit DNMT1	DNMT1	Esophageal Cancer↓	[187]
		WNT5A, TGF-α, BTG2 and MYCBP	Chordoma ↑	[188]
miR-155	C/EBPβ, HDAC4, PPARγ, GLUT4, IRS1, PPAR-c, Creb1, Cebpb, Pparg, Pnpla2, Fabp4, Fasn, AdipoQ,	TNFα, NF-kB pathway, ERK pathway, Caspase 3	Osteosarcoma ↑	[189] [190]
		RhoA, PEG10 and MYB	Breast cancer ↑	[191,192]
			Gallbladder cancer ↑	[193]
		NDFIP1	Melanoma ↑	[194]
		FGF2	ESCC ↑	[195]
			Nasopharyngeal carcinoma ↑	[196]
		IGF-1	Colon cancer ↑	[197]
181c-3p	PPARα; reduced inhibition of PPARα, BC proliferation	PPARα	Breast cancer ↓	[77]
		PTEN	Breast cancer ↑	[198]
		PTEN/PI3K/pAkt	CRC ↓	[199]
		PTEN/PI3K/AKT	NSCLC ↓	[200]
		XIAP, caspase 9, caspase 3	Gastric cancer ↓	[201]
		CTGF, *BIRC5*, *BLC2L1*	Pancreatic cancer ↑	[202]
		MGMT	Glioblastoma ↓	[203]
		SPP1	HCC ↓	[204]
		SMAD7, TGF-β	Osteosarcoma ↓	[205]

Table 1. *Cont.*

miRNAs	Target Genes and Functions in Obesity/IR/MeS	Target Genes or Pathway in Cancer	Type of Cancer (Oncomir/Tumor Suppressor)	References for Cancer Genes or Pathway
MiR-193b	CCL2, NTFYα si NRIP1	DDAH1	Triple-negative breast cancer ↓	[206]
		TGF-β, SMAD3, NF1	CRC, Glioma, Head and neck SCC↑	[207]
		K-Ras, ERBB4	Lung cancer↓	[207]
		MAX, KRAS, TGF-β, CCND1, ETS1, MAPK	ESCC, Gastric cancer, HCC, Pancreatic cancer ↓	[207]
		Mcl-1, c-kit, MYB	Melanoma, Leukemia ↓	[207]
		caspase 3 and 7, uPA	Ovarian, Prostate cancer ↓	[207]
miR-210	NDUFA4 GPD1L	LOXL4	Lung adenocarcinoma ↑	[208]
			Glioma ↑	[209]
			Osteosarcoma ↑	[210]
		HOXA 9	Pancreatic cancer ↑	[211]
miR-221	SIRT1 IRS/PI3K/AKT	PTEN/TRAIL	Breast cancer↑	[212,213]
		ERα, PR, HIF1-α, SLUG	Endometrial cancer↑	[214]
			Prostate cancer ↑	[98],
		MBD2	OSCC ↑	[215]
		Kit	NSCLC ↑	[216]
		PTEN, PPP2R2A	Osteosarcoma↑	[217,218]
		AKT/ERK pathway	HCC ↑	[100]
miR-222	CXCR4 GLUT4 ERs, BTG2, adipor1	p27 (kip1)	NSLCC	[216]
		MST3	CRC ↑	[219]
		PPP2R2A	Papillary thyroid cancer ↑	[220]
miR-221/222	CAV1	CAV1	Breast cancer↑	[51]
		β4 integrin, STAT5A, and ADAM-17	Breast cancer↑	[221]
		p27, p57, ER∝	Breast cancer↑	[222]
		Wnt/β-catenin, WIF1, SFRP2, DKK2, AXIN2	Breast cancer↑	[223]
		TIMP2	Gliomas ↑	[224]
			Retinoblastomas ↑	[225]
miR-302	Maintain SOX2 and c-Myc by targeting repressor of c-Myc	MACC1	HCC ↓	[83]
		Sox2, c-Myc, Nanog	Breast cancer ↑	[226]
		RUNX2	Breast cancer ↓	[227]
		TGF-β	Mucoepidermoid carcinoma of salivary glands ↑	[228]
		TGFBR2/SMAD3 RAB11A/Wnt/β-Catenin	Pituitary Tumors ↑	[229]

Table 1. *Cont.*

miRNAs	Target Genes and Functions in Obesity/IR/MeS	Target Genes or Pathway in Cancer	Type of Cancer (Oncomir/Tumor Suppressor)	References for Cancer Genes or Pathway
miR-365	Cebpα, Fabp4, and Pparγ	BTG2	Pancreas ↑	[230]
		ETS1	NSCLC ↓	[231]
		ADAM1	Triple negative breast cancer ↓	[232]
miR-375	ERK $\frac{1}{2}$ Myotrophin	PSAT1	ESCC ↓	[233]
		AEG-1	HCC, Head and neck cancers ↓	[234,235]
		PDK1, YWHAZ	Gastric ↓	[236]
miR 3184-3p	FOXP4–NOTCH induced EMT	N-cadherin, vimentin, E-cadherin	Breast cancer ↓	[77]
Let 7	Inhibit HMGA2, inhibit preadipocyte proliferation, insulin-PI3K-mTOR IGF1R, INSR, IRS2	HMGA2	Breast cancer ↓	[302]
		lin-41, hbl-1/lin-57 RAS	Lung cancer ↓	[229,237,238]
		KDM3A/DCLK1/FXYD3	Lung cancer ↓	[239]
		HGMA2	Lung cancer ↓	[240]
		RAS, c-MYC	CRC ↑	[241]
		HGMA2 LIN 28	CRC ↓	[242–244]
		E2F2, CCND2	Prostate cancer↓	[245]
		RAS	Ovarian cancer ↓	[246]
		MYCN	Neuroblastoma ↓	[247,248]
		Aurora-B	Osteosarcoma ↓	[249]

The increased expression of oncomiR-155 in the osteosarcoma cancer cells was associated with the upregulation of several cancer stem cell surface markers, transcriptional factors, and Actinomycin D treatment resistance. Additionally, a positive feedback loop was identified between TNF-alpha and miR-155 which resulted in increased cancer cells aggressiveness [189]. MiR-155 downregulation suppresses cell proliferation and leads to cell programmed death via the NF-kB pathway [190].

OncomiR-155 plays a part in the breast cancers' pathophysiological process. Thus, miR-155 was correlated with tumor-associated inflammation, metastatic adenopathy [191], and aggressiveness [192].

The overexpression of oncomiR-155 in gallbladder cancer was demonstrated and proved to be a marker of tumors' aggressiveness and unfavorable prognosis being associated with cancers' progression and lymph-node metastasis [193].

The implication of miR-155 was also observed in other types of cancers, for example: uveal melanoma via Nedd4-family interacting protein 1 [194], esophageal cancer via FGF2 [195], nasopharyngeal carcinoma [196], and colon cancer [197].

A common target of mir-155 that links MetS/obesity and cancer is *PPARg*. *PPARg* mediates the mir-155 effect on adipose cells. Thus, mir-155, by targeting the PPAR mRNA 3′UTR, regulates the chemokine expression in adipocytes, the overexpression of mir-155 being associated with the adipose tissue inflammation [282]. Furthermore, the downregulation of *PPARg* disrupts the metabolic homeostasis in adipose tissue and favours the beige/brown differentiation of fat cells. More important is that the upregulation of *PPARg* proved to be helpful in combating breast cancer-associated cachexia [304].

MiR-193b

High values of miR-193b were detected in the serum of persons with IR compared to the serum of subjects with preserved insulin sensitivity, as well as in patients with prediabetes compared to those with T2D, suggesting the utility of this biomarker in the early diagnosis of these disorders [305,306]. MiR-193b is expressed in high amounts in adipose tissue, where it contributes to the differentiation of brown adipocytes and to the decrease of inflammation through its inhibitory action on CCL-2 (chemokine C-C motif ligand 2), a key factor involved in inflammation associated with obesity. Low MiR-193b expression was found in subcutaneous adipose tissue of people with obesity [177].

Regarding its implication in carcinogenesis, miR-193b seems to play a dual role, acting as an oncogene in some types of cancers and as a tumor suppressor in others [207].

Several molecular pathways have been proposed to explain the pathogenic role of miR-193 in cancer. For example, in triple negative breast carcinoma, there is a low expression of miR-193b, which is correlated with a high expression of its target DDAH1, a protein with an important role in tumor angiogenesis [206]. In CRC and gastric adenocarcinoma, miR-193 acts on the TGF-beta signaling pathway, with a role in cell proliferation and apoptosis. In SCCHN, miR-193b acts as an oncogene through its action on NF1, and in pancreatic cancer, it acts as a tumor suppressor by directly targeting KRAS through AKT, ERK, and MAPK pathways [207].

MiR-181c-3p

MiR-181c-3p is a member of the miR-181 family and has been considered to be a novel tumor-associated miRNA in recent years. The role of miR-181c in cancer progression is controversial [77].

MiR-181c commonly appears to be a suppressing factor in various malignancies. In gastric cancer, miR-181c is significantly down-regulated and correlates with a relatively poor prognosis [201]. In breast cancer, miR-181c is the up-streaming regulator of PPAR-α implicated in EMT, being remarkably decreased in cancer cells [77]. Down-regulation of PPARα was significant, while expression of miR-181c-3p was induced by ectopically using miR-181c-3p mimic. Based on our study, miR-181c-3p would be considered as a tumor suppressor miRNA, and *PPARα* as a direct target gene for miR-181c-3p.

According to Wang, *SPP1* is one of the genes likely to participate in the enhancement of HCC growth, which provides a new potential target for the prevention and treatment of HCC. Furthermore, miR-181c in HCC cells presents characteristic direct interaction with *SPP1* as an up-streaming inhibitor, which strongly suggests new strategies in HCC research and treatment for establishing interventional practice at the molecular level.

Zhang et al. demonstrated the role of miR-181 oncomir by suppression of *PTEN* in breast cancer [198]. In pancreatic cancer, miR-181c is significantly increased [198].

Liu et al. demonstrated that overexpression of miR-181 in A549/DDP cells induced apoptosis and autophagy, reducing cell proliferation and migration via the PTEN/PI3K/AKT/mTOR pathway [307]. Thus, miR-181 could be useful in elucidating the potential molecular mechanisms underlying chemotherapy drug resistance in NSCLC, providing a foundation for novel therapeutic strategies for the treatment of NSCLC in the clinical setting.

MiR-210

In patients suffering from obesity, mir-210 is overexpressed in ATM [283]. Moreover, miR-210 resulted from ATM is implicated in the pathogenesis of diabetes in obese mouse models by altering glucose uptake and mitochondrial complex IV activity by targeting NADH dehydrogenase ubiquinone 1 alpha subcomplex 4 gene [308].

The mir-210 disrupted mitochondrial metabolic functions might also be implicated in cancer. GPD1L is a direct target of mir-210, which is implicated in mitochondrial homeostasis in hypoxic conditions. A valid hypothesis is that mir-210 might adjust its functions according to the oxygenation of the tumor microenvironment. Thus, it might act as a tumor suppressor in the initial stages of tumor growth and as an oncomir as the cancerous process evolves and the hypoxia becomes more important [309].

MiR-210 exerts its effects in lung adenocarcinoma cells by targeting Lysyl oxidase-like 4 (LOXL4). Consequently, the tumor cells exhibit enhanced capacities of proliferation, migration, and invasion [208].

Oncomir-210 is a marker for poor prognosis in gliomas [250] and its overexpression was demonstrated in osteosarcoma cells [210].

In pancreatic cancer cells, an increased expression of mir-210 under hypoxic conditions can be noticed. Additionally, hypoxia conditions cause gemcitabine treatment resistance and epithelial–mesenchymal transition [211].

MiR-221

Decreased plasmatic levels of miR-221 in obese subjects have been described in several studies [310,311]. Furthermore, reduced levels of miR-221 were associated with gestational obesity [312]. The pattern of miR-221 expression among morbidly obese patients changed once the weight loss surgery was performed. Thus, the levels of miR-221 were upregulated in patients that underwent gastric-bypass surgery [310]. The dysregulation of circulating miRs in obesity and diabetes has been further assessed by Nunez Lopez et al. [313].

By reducing Sirtuin-1 (SIRT1) protein levels, miR-221 induces white adipose tissue inflammation and IR [314]. Palmitic acid upregulated the expression of miR-221 which consequently disturbed the IRS/PI3K/AKT signaling in the initial phases of IR [42].

MiR-221 could be responsible for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance in breast cancer cells by regulating *PTEN*, thus inducing EMT and increasing migration and invasiveness of breast cancer cells. Interestingly, TRAIL sensitization and a reduction of migration abilities and invasiveness of the neoplastic cells were noticed after oncomir-221 knockdown [212]. MiR-221 is implicated in trastuzumab resistance of the HER2 -positive breast cancer cell line by targeting *PTEN*. On the other hand, the *PTEN* overexpression reversed the trastuzumab resistance in breast cancer cells and suppressed their invasion capacities [213].

The link between estrogen-receptor-alpha (ERα), progesterone receptor (PR), hypoxia-inducible factor 1-alpha (HIF1-α), SLUG, and miR-221 circuit was also investigated in obese and nonobese women diagnosed with endometrial cancer [214], but further studies are needed in this regard.

OSCC invasion and migration were augmented by the direct interaction of miR-221 and its target 3-UTR of methyl-CpG binding domain protein 2 (MBD2) with a consequent reduction of the MBD2 protein [215].

The profile of miRs regulating the TNF-related apoptosis-inducing ligand (TRAIL) in NSCL revealed that TRAIL resistant cells overexpressed five miRs among which are miR-221 and -222. TRAIL-induced apoptotic cell death proved to be mediated by the oncomirs-221 and -222 and their target 3-UTR of Kit and p27 (kip1) mRNAs [216].

The overexpression of miR-221 in osteosarcoma cells increased their aggressiveness not only by enhancing their proliferation and migration abilities but also by augmenting their invasiveness. The underlying mechanism that may explain this process is PTEN suppression [217]. MiR-221 is responsible for cisplatin resistance in osteosarcoma cells through protein phosphatase 2 regulatory subunit B alpha (PPP2R2A) downregulation [218].

The tumor microenvironment plays a decisive role in cancer progression and metastasis as shown in the study of the interactions between extracellular vesicles (EVs)-derived oncomirs, HCC cells, and cancer-associated hepatic stellate cells (caHSCs). In this regard, miR-221 together with miR-21 and -151 proved to have an oncogenic effect on HHC cells by modulating the protein kinase B (AKT)/extracellular signal-regulated kinase (ERK) pathways [100].

The oncomiR-221 upregulation is associated with the gastric cancer' progression, invasiveness, lymph node metastasis, and an overall poor prognostic [315].

Regarding prostate cancer, there is a cluster of miRs (miR-20a, miR-21, miR-145, and miR-221) which proved to be helpful in the differentiation between high risk and low risk patients concerning the aggressiveness of prostate cancer [89]. Interestingly, the miR-221 expression in prostate cancer patients is reduced by the AR agonists (mibolerone (MIB)

and dihydrotestosterone (DHT)) [316]. This unpredictable effect should be evaluated in future studies in order to establish the clinical implications of these findings.

SRT1 represents the common pathway that links cancer and IR/adipose tissue inflammation. Thus, an increased expression of mir-221 not only decreases the SIRT1 protein level resulting in IR and inflammation in adipocytes [314] but also regulates the prostate cancer progression. Even though SIRT1 does not represent a direct target of mir-221, in prostate cancer cell transfected with mir-221, the inhibitor SIRT1 protein was up-regulated [317].

MiR-222

MiR-222 is not only overexpressed in adult patients with obesity [310], but also in obese children and adolescents [289,318]. Moreover, the alterations in the expression of miR-222 in those patients are associated with imbalances of MetS biological markers [286]. T2D was also associated with disturbances in the circulating levels of miR-222. Noteworthy is the fact that insulin infusion reduced the circulating levels of miR-222. Furthermore, miR-222 levels were inversely correlated with the metformin dose administrated in T2D subjects [319]. De Mendonça M. et al. found that miR-222 mediates the effect of pioglitazone on insulin sensitivity in skeletal muscle of diet-induced obese mice, independent of PPAR [320].

In placenta and/or pancreatic tissues of patients and animal models with gestational diabetes (GDM), the expression of miR-222 and NLR family pyrin domain containing 3 (NLRP3) inflammasomes were up-regulated while C-X-C chemokine receptor type 4 (CXCR4) was downregulated. Improvement of insulin sensitivity in GDM mice through the inhibition of miR-222 together with the overexpression of CXCR4 was noticed. The up-regulation of miR-222 together with the downregulation of glucose transporter 4 (GLUT4) and estrogen receptors (ERs) were strongly correlated with the serum concentration of estradiol. Thus, the hypothesis that the action of miR-222 on GLUT4 and ERs is responsible for the estrogen-induced IR [321].

MiR-222 promotes the proliferation of preadipocytes and the accumulation of lipids in mature adipocytes by inhibiting the lipolysis. Increasing evidence links miR-222 to MetS, making it a valuable potential therapeutic target in the management of obesity and IR [322].

The mir-222/CXCR4 pathway is not only implicated in GDM but also in breast cancer. Mir-222 expression was downregulated in breast cancer-associated TAMs. Mir-222 regulates the macrophage migration in breast cancer through the CXCR4 pathway. Mir-222 is inversely correlated with TAM chemotaxis [323]. OncomiR-222 overexpression proved to increase colon cancer cell aggressiveness by promoting their migration and invasion abilities. MiR-222 alters the colon cancer cell migration through the downregulation of its target gene mammalian STE20-like protein kinase 3 (MST3) which plays a key role in the phosphorylation of paxillin, thus reducing the intercellular adhesion. Furthermore, miR-222 together with MST3 play a crucial role in the production of inadopodia [219]. Furthermore, the overexpression of miR-222 in aggressive papillary thyroid cancer tissues was established. In vitro studies revealed that miR-222 exerts its effects via 3′-UTR of protein phosphatase 2 regulatory subunit B alpha (PPP2R2A), thus enhancing the invasiveness and the migration of thyroid cancer cells. The AKT signaling pathway also proved to play a role in miR-222-mediated invasion and metastasis of papillary thyroid cancer [220]. As in colon cancer, miR-222 proved to enhance the formation of lung metastasis in thyroid cancer patients [219,220].

Mir-221/222

The miR-221/222 cluster is implicated in both IR and breast cancer pathogenesis through its downregulating effect on CAV1. Mir-221/222-induced deregulation of CAV1 represents a key pathway involved in breast cancers' invasion, migration, and metastasis [51].

Furthermore, the cluster mir-222/221 is implicated in both IR and cancer by targeting genes that are implicated in both MetS and cancer pathogenesis: *transcription factor v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1), DICER, PTEN* [324].

Regarding the implication of miR-221/-222 in the cancers' pathological process, it targets the following genes *p27Kip1, CDKN1C/p57, E-cadherin, PTPμ, PUMA, ARID1A, AHR1* [324].

Cancer lack of responsiveness to treatment remains an ongoing impediment for clinicians. The aggressiveness of this disease is in part the result of miR-221/-222-mediated signaling by targeting β4 integrin, STAT5A, and ADAM-17 [221]. Thus, the role of the miR-221/-222 cluster and its target genes (*p27, p57, estrogen receptor alpha*) in BC cells survival and the lack of response to estrogen was established [222]. The increased expression of miR-221/-222 in breast cancer cells enhances tumor aggressiveness through the activation of Wnt/β-catenin signaling by downregulating the target genes *WIF1, DKK2, SFRP2,* and *AXIN2* [223].

Deregulation of miR-221/-222 is also implicated in other solid malignancies such as gliomas, by targeting TIMP2 [224] and retinoblastomas [225].

MiR-365

Along with miR-193b, miR-365 plays an important role in differentiation of brown adipocytes. Recently, it has been demonstrated that brown adipose tissue (BAT) plays a more important role in humans than was initially considered. The amount of BAT is inversely correlated with BMI and the basal metabolic rate. The decrease in BAT activity may contribute to the development of obesity and IR. In vitro inhibition of miR-193a/b and miR-365 expression inhibits brown adipocyte differentiation as a result of the inhibition of some key genes involved in adipogenesis such as *adiponectin*, *Cebpα*, *Fabp4*, and *Pparγ* [325].

In pancreatic ductal adenocarcinoma, miR-365 values were associated with the therapeutic response [230]. In NSCLC, the values of miR-365 are correlated with prognosis, and this is involved in tumor pathogenesis through its action on TTF1, ETS1, PTEN [231]. In triple-negative breast cancer, miR-365 inhibits tumor proliferation, migration, and invasion through its action on ADAM1, the miR-365/ADAM1 axis being suggested as a possible therapeutic target [232].

MIR-375

MiR-375 is one of the miRs specific to pancreatic beta cells with a role in the suppression of glucose-stimulated insulin secretion through inhibition of myotrophin expression. Additionally, it plays an important role in glucose homeostasis, cell turnover, and in the differentiation of pancreatic beta cells [326]. In addition, it stimulates adipogenesis in preadipocytes by regulation of the ERK1/2 signaling pathway [327].

Some studies showed a significant increase in miR-375 in the plasma of patients with T2D compared to normoglycemic persons, suggesting its potential utility as a biomarker [272].

Numerous studies demonstrated the implication of miR-345 in the process of carcinogenesis. For example, miR-375 inhibits AEG-1 oncogene expression, and low miR-375 values accompanied by AEG-1 overexpression are involved in tumor growth and invasion in HCC and head and neck cancers [234,235]. In gastric cancer, low expression stimulates cell proliferation by attenuating the effect on the JAK2 signaling pathway and through the action on PDK1 and YWHAZ [236]. The tumor suppressive effect of miR-365 in esophageal squamous cell carcinoma was demonstrated in vivo and in vitro by identifying IGF1R as a target of miR-365, an important component of the PI3K-AKT/PKB pathway. Other cancers in which a low miR-365 expression was observed are uterine cervical cancer through the action on SP1, prostate cancer, CRC, melanoma, pancreatic cancer [233].

Let-7

Let-7 is downregulated in obesity and it targets HMGA2 [328]. The let-7 family is involved in adipocyte differentiation by targeting the high-mobility group AT-Hook 2 (HMGA2) protein, which reduces adipose tissue in obese leptin-deficient mice [329], suggesting, once again, a role for leptin and obesity in CRC [330].

In breast cancer, many studies have shown that let-7 inhibits HMGA2, MYC, JAK-STAT-3, caspase-3, RAS, CCND2, Erα [331–333].

In lung cancer, let-7 miRNA expression levels are changed [237] and low let-7 expression is significantly associated with shorter postoperative survival. In contrast, the

study of Inamura [238] shows that decreased expression of let-7 occurs early during tumor progression and does not correlate with prognosis of bronchioloalveolar carcinoma. In lung cancers, up-regulation of HMGA2 and down-regulation of let-7 has been reported [240]. The effect of let-7 on HMGA2 was determined by multiple target sites in the 3′ untranslated region (UTR), and overexpression of the HMGA2 ORF without a 3′UTR rescued the growth-suppressive effect of let-7 on lung cancer cells. These results offer a novel example of suppression of an oncogene by a tumor-suppressive miRNA and indicate that the oncogene is activated by some tumors through chromosomal translocations that remove the oncogene's 3′UTR containing the let-7 target sites.

Overexpression of let-7 has been shown to inhibit proliferation of ovarian cancer [246], prostate cancer [245], colon cancer [242], osteosarcoma [249], and neuroblastoma [247]. Several important cell cycle regulators including cyclins, cyclin-dependent kinases (CDKs), Ras, HMGA2, *MYCN*, and c-Myc have been confirmed to be targets of let-7 (see Table 1).

Let-7 functions as an onco-miR in CRC [241].

The important roles played by LIN28/let-7 in tumor progression involve this pathway as an attractive therapeutic target. Reversal of LIN28 expression in a full-blown tumor has been demonstrated to induce tumor cell differentiation and decreased tumor invasiveness, and antagonizing LIN28 would induce tumor cell differentiation and might have beneficial effects alongside chemotherapy, given that well-differentiated tumors are generally less aggressive and less drug-resistant, having better clinical outcomes [245]. He et al. [243] showed that the *PVT1-214*/Lin28/let-7 axis performs the function of a critical regulator of CRC pathogenesis, which may provide a new direction for the development of CRC therapy.

3. Conclusions

Obesity/Mets can induce cancer by deregulation of several miRs that are involved in metabolic processes, inflammation, and proliferation signaling. On the other side, different miRs are deregulated in cancer patients with comorbid obesity/MS, suggesting that there are some sharing mechanisms involved in adipogenesis and carcinogenesis. Currently, there is no single miR that can predict the prognosis or serve as a single biomarker. Some combinations of miRs have the potential to become prognostic markers, specific to different types of cancer, but this possibility needs to be further explored and validated.

Based on the tight connection between cancer and inflammation, targeting the inflammatory factors of the tumor microenvironment is a promising strategy for cancer prevention and treatment.

Modulation of these miRs with mimics or inhibitors could serve as a promising cancer gene therapy for tumor control and metastasis inhibition.

A variety of dietary compounds and supplements found in cruciferous vegetables, green tea, soya, turmeric, red grapes, blueberries, and spices like curry and black pepper proved beneficial in cancer prevention by modulation of microRNAs [334]. They are able to modify the epigenome and can be incorporated into the 'epigenetic diet' to protect against cancer and the aging process.

Author Contributions: Conceptualization, A.F., A.C., A.L.L. and C.B.; methodology, O.H.O. and B.T.; software, O.H.O. and B.T.; validation, A.F., A.C., A.L.L. and C.B.; formal analysis, B.T.; investigation, O.H.O.; resources, B.T.; data curation, O.H.O.; writing—original draft preparation, A.F., A.C., A.L.L. and C.B.; writing—review and editing, A.F., A.C., A.L.L. and C.B; visualization, B.T.; supervision, A.C.; project administration, A.F. All authors have read and agreed to the published version of the manuscript.

Funding: The research received no external funding. The APC was funded by authors and University of Medicine and Pharmacy.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Received: 17 October 2020 | Revised: 28 November 2020 | Accepted: 5 December 2020

DOI: 10.1111/eci.13475

REVIEW ARTICLE

WILEY

Assessment of subclinical diabetic cardiomyopathy by speckle-tracking imaging

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Abstract

Background: Diastolic dysfunction is traditionally believed to be the first subclinical manifestation of diabetic cardiomyopathy (DCM), leading to systolic dysfunction and then overt heart failure. However, in the last few years, several studies suggested that systolic subclinical dysfunction measured by speckle-tracking echocardiography (STE) may appear ahead of diastolic dysfunction. In this review, the main endpoint is to show whether subclinical myocardial systolic dysfunction appears ahead of diastolic dysfunction and the implication this may have on the evolution and management of DCM.

Materials and methods: We performed a search in PubMed for all relevant publications on the assessment of DCM by STE from 1 June 2015 to 1 June 2020.

Results and Conclusions: The results illustrate that subclinical systolic dysfunction assessed by STE is present in early DCM stages, with or without the association of diastolic dysfunction. This could be a promising perspective for the early management of patients with DCM leading to prevention of the overt form of disease.

KEYWORDS

diabetic cardiomyopathy, diastolic dysfunction, speckle-tracking echocardiography, strain, subclinical, systolic dysfunction

1 | INTRODUCTION

The relationship between diabetes mellitus (DM) and heart failure (HF) was first suggested in 1881 by Leyden, who stated that HF is a 'frequent and noteworthy complication of DM'.[1] Diabetic cardiomyopathy (DCM) was described for the first time by Rubler et al in 1972 in their manuscript entitled 'New type of cardiomyopathy associated with diabetic glomerulosclerosis', the authors reporting the presence of HF in diabetic patients without other cardiac comorbidities.[1-4] After 4 decades, the myth has become reality; now, we are able to define DCM as the presence of ventricular dysfunction in the absence of hypertension, coronary artery disease, congenital or valvular heart disease.[2,3,5]

The Framingham study evidenced a 2- to 5-fold increase in the risk of developing HF in patients suffering from DM and an even higher risk in young DM patients.[2] DM itself is responsible for 1.5 million deaths every year, most of them caused by cardiovascular diseases, being an independent predictor of mortality and HF with preserved left ventricular (LV) ejection fraction (HFpEF).[3] Furthermore, asymptomatic LV dysfunction, either diastolic or systolic,

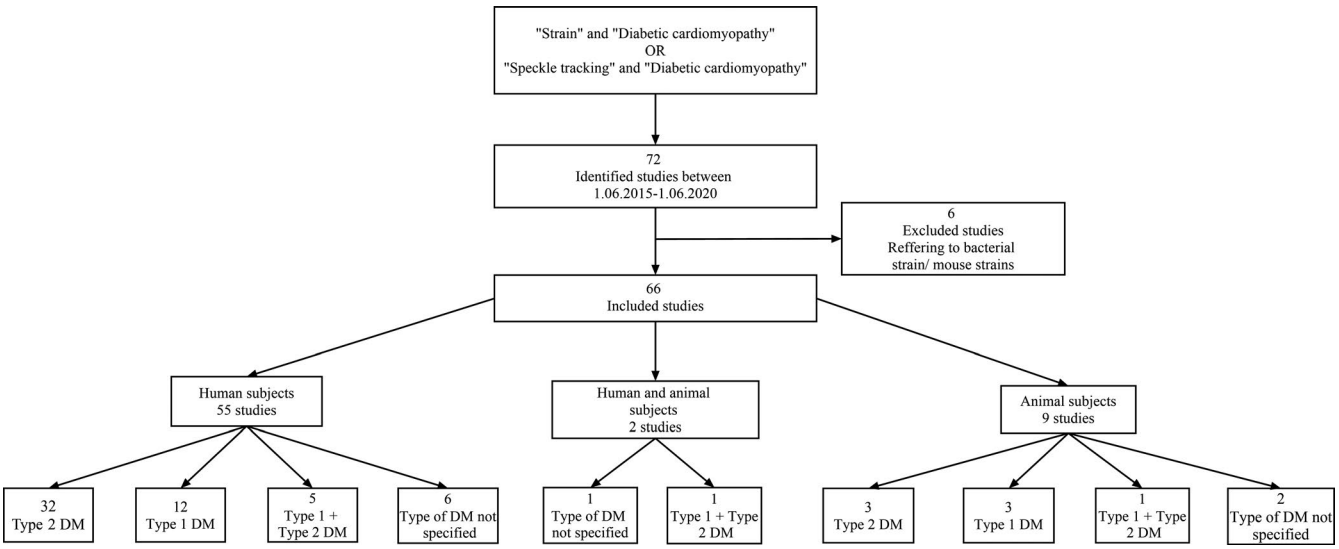

FIGURE 1 Article selection protocol. DM, diabetes mellitus

was found to be present in 50%-70% of patients suffering from DM.[6]

The exact mechanism that leads to DCM is incompletely understood, but most of the studies suggest hyperglycaemia with hyperinsulinemia and insulin resistance as the starting points of the cascade leading to DCM.[2,7,8] Consequently, multiple metabolic pathways are activated: the sympathetic nervous system causing autonomic dysfunction, the renin-angiotensin-aldosterone system inducing pro-hypertrophic and pro-fibrotic changes, oxidative stress with the formation of reactive oxygen species and consequent mitochondrial dysfunction and calcium homeostasis impairment.[2] All these mechanisms result in myocardial fibrosis and hypertrophy, cardiomyocyte death and coronary circulation impairment, leading to HF and DCM.[2,7-9] There are certain pathophysiological differences between type 1 and type 2 DM regarding the trigger of DCM: in type 1 DM, the principal trigger was found to be hyperglycaemia, whereas in type 2 DM, hyperinsulinemia and insulin resistance are the main triggers.[8] Early hyperglycaemic status in type 1 DM is associated with delayed calcium uptake by the sarcoplasmic reticulum, leading to impairment of LV contractility and active relaxation, causing both systolic and diastolic dysfunction.[10] In contrast, hyperinsulinemia and later insulin resistance in type 2 DM patients are associated with fibrotic LV remodelling, resulting in increased stiffness, thus inducing predominantly diastolic myocardial dysfunction in the early stages of disease.[11]

Speckle-tracking echocardiography (STE) is a relatively new technique that can assess subclinical cardiac function by measuring myocardial strain—the deformation of specific areas of the myocardium during cardiac cycle. In the last decade, it has become an essential tool in echocardiography laboratories worldwide, showing higher specificity compared to traditional echocardiography parameters such as left ventricle ejection fraction.[1,12,13]

Most of the studies support the traditional hypothesis that LV diastolic dysfunction is the first cardiac functional alteration in the development of DCM.[1-3,10,14,15] However, several recent studies suggest that subclinical systolic dysfunction measured by STE may occur ahead of diastolic dysfunction,[5,8,16,17] or at least concomitantly with it.[4,7,11,18,19] Therefore, detecting ventricular dysfunction as early as its subclinical stages could be an important step in order to lower the morbidity and mortality in patients with DM.[3]

2 | METHODS

In order to identify all relevant publications, the authors searched PubMed using the following keyword combinations: "speckle-tracking" AND "DCM" OR "strain" AND "DCM". All articles including DCM and strain or speckle-tracking, published in English over the last 5 years—1st June 2015 - 1st June 2020, were retained. Both human and animal studies were included. The initial search in June found 72 studies, of which 6 were excluded. A total number of 66 articles were selected (see Figure 1 for a more detailed selection protocol). A narrative review was then composed to present the results. All studies, both on human or animal subjects, included the appropriate statements regarding research protocol approval, the former providing human informed consent when necessary.

Reporting of the study conforms to broad EQUATOR guidelines (Simera et al January 2010 issue of EJCI).

3 | RESULTS AND DISCUSSION

A list of all 66 studies including demographics and parameters used for myocardial function assessment is summarized in Table 1.

3.1 | Cardiac response to diabetes mellitus

The pathophysiology of the structural and functional changes leading to myocardial dysfunction in DM patients is complex. Metabolic alterations such as hyperglycaemia, insulin resistance and hyperinsulinemia are the main factors that induce myocardial dysfunction.[1,2]

Going further into the pathophysiological chain of DCM, low insulin sensitivity and glucose assimilation in cardiomyocytes caused by the impairment of certain glucose transporters stimulate fatty acid degradation in order to compensate the necessary cardiac amount of energy.[1] However, fatty acid metabolism modifies intracellular signalling in cardiomyocytes, leading to inefficient energy production and cardiac lipotoxicity through the generation of reactive oxygen species and other secondary end-products such as ceramide and diacylglycerol.[1,2,4] Moreover, hyperglycaemia stimulates the production of advanced glycation end-products which also play a key role in the activation of pro-oxidant and pro-inflammatory response.[1] All of these promote impaired calcium homeostasis, mitochondrial/endoplasmic reticulum failure, renin-angiotensin system enhancement, autonomic neuropathy and apoptosis, finally leading to chronic inflammation, fibrosis and cardiomyocyte relaxation-contractility dysfunction, as part of DCM.[1,2,4,10]

Furthermore, it was shown that subclinical myocardial dysfunction may even occur in pre-diabetes patients, suggesting that dysglycaemia, and not necessarily hyperglycaemia, may induce the pathophysiological alterations leading to DCM.[20]

For a better understanding of DCM pathophysiology, it is important to understand the principal types of cardiac dysfunction. Diastolic dysfunction is characterized by a defect in ventricular relaxation leading to impaired ventricular filling and high filling pressures during diastole, while systolic dysfunction is defined as the inability of the myocardium to pump an adequate amount of blood during systole. In early DCM stages, diastolic dysfunction identified as reduced diastolic filling pressures, prolonged isovolumetric relaxation and increased atrial filling pressures is already established, while systolic dysfunction traditionally assessed by LV ejection fraction is absent.[1] However, more specific parameters assessed by STE demonstrated the presence of systolic dysfunction, in its subclinical form, in the early stages of DCM.[4,10,11]

3.2 | General principles of speckle-tracking echocardiography

STE is a relatively new technique for myocardial mechanical deformation assessment, allowing a more objective and quantitative evaluation of myocardial function.[1,12,13] It is based on tracking specific speckle patterns created by interferences of ultrasound beams in the myocardium, providing advanced systolic and diastolic function measurement. Strain and strain rate are myocardial deformation parameters which, despite having initially been integrated in Doppler imaging, have acquired a more important role through STE. They measure the degree of deformation of a specific area of the myocardium during a cardiac cycle, in relation to its initial dimensions (strain), and the speed of this deformation (strain rate). The strain (ε) equation is $\varepsilon=(L-L_0)/L_0$, where L_0 stands for basal length and L for length after deformation of the area of interest. The results are based on the shortening and lengthening of the measured area, the outcomes being negative values for the former and positive values for the latter.[12,13] STE measures myocardial deformation in 3 axes (longitudinal, circumferential and radial strains).[1,12,13] Longitudinal strain represents the myocardial deformation from the base to the apex, radial strain the deformation radially directed towards the centre of the LV cavity, and circumferential strain the shortening along a circular area (Figure 2).[12,13] Images are obtained during breath holding with stable electrocardiographic tracings, and the measurements of global and regional deformation are performed offline. Global longitudinal strain (GLS) has been validated for the assessment of global LV function.[12]

3.3 | Echocardiographic assessment of diabetic cardiomyopathy

3.3.1 | Left ventricular myocardial dysfunction assessment

The most widespread approach to LV function assessment is traditional echocardiography. However, in the last decade, modern echocardiography technologies such as tissue Doppler imaging and STE have gained ground in myocardial function assessment.[1] Although tissue Doppler imaging is the predominant technique for assessing LV diastolic function, when it comes to LV systolic function and myocardial deformation measurement, it has a series of disadvantages: setting up a specific volume of the sample to measure annular velocity,[21] inter/intra-observer variability, angle dependence or noise interference,[1,8] STE, on the other hand, overcomes the limitations of Doppler techniques, providing a comprehensive analysis of both LV systolic and diastolic functions.[1,14] A comparison of STE with other imaging modalities in terms of principal advantages and limitations is showed in Table 2.

TABLE 1 Demographics and parameters of myocardial dysfunction used in reviewed articles

Year	Study	Age	No of patients	Subclinical Systolic Dysfunction	Diastolic Dysfunction	Technique
2020	Gao Y et al	50.95 ± 10.185/ 54.85 ± 10.87/ 54.48 ± 9.61	73	LVLS, LVCS, LVRS,	-	CMR
2020	Chen X et al	50.08 ± 6.01/ 52.35 ± 8.25/ 53.15 ± 7.50	117	GLS, GCS, GRS GAS	E, A, E/A	3D-STE, PWD
2020	Pappritz K et al	mice	-	GLS, GCS	-	STE
2020	Tadic M et al	Review	-	-	GLS, GCS	CMR
2020	Mohseni-Badalabadi R et al	61.0 ± 8.1/58.4 ± 8.6	96	LASr, LAScd, LASct, pLASRr, pLASRcd, pLASRct,	E/A, E/E', A, E, E', S, D, S'	2D-STE, PDW, TDI
2020	Shao G et al	54.1 ± 7.5/ 56.8 ± 6.5/ 54.1 ± 6.2	84	GLS, LACS, LARS, LALS, RVLS, LVRS, LVCS, LVLS	-	CMR-FT
2019	Jensen MT et al	61.3 (7.5)/ 61.2 (7.5)/ 63.5 (7.0)	3984	GCS basal, GCS mid, GCS apical	-	CMR
2019	Athithan L et al	Review	-	GCS, GLS, PSSR	PEDSR	CMR, TDI
2019	Paiman EHM et al	59.5 ± 6.6/57.6 ± 7.8, 51.3 ± 9.0/48.3 ± 8.1	131	GLS, GCS	GLSR-E, GCSR-E E/A ratio	CMR
2019	Vukomanovic V et al	50 ± 9/52 ± 8	136	LA longitudinal strain parameters, LA global strain, LA positive strain, LA negative strain		2D-STE, TDI
2019	Iwakura K	-	-	GLS	E/E'	2D-STE, TDI
2019	Zhang Y et al	rhesus monkeys	27	GLS	E/A, E/E', E'/A', GSrL	TTE, CMR
2019	Huang J et al	Not specified	103	LS-endo, LS-mid, LS-epi, LSr, PSLR	E/A, E/E'	2D STE, PWD, TDI
2019	Obaid N et al	63 + 7/55 + 11	90	GLS	E, E', A, E/A, E/E'	2DSTE, PWD, TDI
2019	Iso T et al	10.2 (5.3-14.8)/ 20.4 (15.2-24.7)/ 33.4 (27.9-40.3)/ 10.2 (5.4-14.5)/ 20.4 (15.7-24.8)/ 31.9 (26.2-40.7)	130	GLS: -endocardial -midmyocardial -epicardial GCS: -basal -papillary -apical	E, A, E/A, E', E/E'	2D-STE, PWD, TDI
2019	Van Berendoncks AM et al	46	102	GLPSS		2D-STE, CT for CACS
2019	Bogdanovic J	Group A: 38.3 ± 1.6 Group B: 43.2 ± 3.6 Group C: 32.2 ± 2	107	GLS, LS-endo, LS-mid, LS-epi	E/A, E/E'	2D-STE, PWD, TDI
2018	Ahmed AT et al	18.2 ± 1.7 18.8 ± 2.3	54	RVLS	RV-E, RV-A, RV-E/RV-A, RV-E', RV-E/RV-E'	2DSTE, PWD, TDI

(Continues)

TABLE 1 (Continued)

Year	Study	Age	No of patients	Subclinical Systolic Dysfunction	Diastolic Dysfunction	Technique
2018	Cameli M et al	62.5 ± 9.9	22	GLS, PALS, GAVS	E, E', E/E',A'	2D-STE
2018	Zhu T et al	Rhesus monkeys	19	Ecc, Ell tEcc, tEll	CSR, LSR	CMR
2018	Liu X et al	53.23 ± 8.59/50.74 ± 11.92/56.50 ± 9.96	101	PSSR: -radial, -circumferential, -longitudinal	PEDSR -radial, -circumferential, -longitudinal	CMR
2018	Tabako S et al	57.0 ± 14.0/50.6 ± 13.6/60.1 ± 13.1	166	GLS, GRS	E, A, E', E/A, E/E'	2D-STE, PWD, TDI
2018	Shang Y et al	51.2 ± 12.1/54.3 ± 7.9	89	PSSR: -radial, -circumferential -longitudinal	PEDSR -radial -circumferential -longitudinal	CMR
2018	Wang Y et al	70.9 _ 4.3	290	GLS	E, A, E' E/A, E/E'	2D-STE, PWD, TDI
2018	S. Costantino et al	Mice	Not specified	GLS	-	Genetic tests, 2D STE
2018	Lin et al	49.7 ± 10.7 years	3950	GLS	E, EDT, E/E'	2D STE, PWD, TDI
2018	Mátyás et al	Rat	28	GCS, SrS	SrIVR, SrE	STE
2018	Zhou et al	Mice	20	GLS, GCS, GRS	E/A, EDT, IVRT	2D STE, PWD
2018	Negishi et al	Review	-	GLS, GCS, GRS	E/A, EDT, E/E'	2D STE, PWD, TDI
2018	Cao et al	55 ± 7/54 ± 6	82	GLS, GCS, GRS	LSR. CSR, RSR	CMR
2017	Theilade et al	54 ± 12	305	GLS	E/E'	2D STE, TDI
2017	Tadic et al	55 ± 9/57 ± 8	146	Multilayer	E/A, EDT, E/E'	2D STE, PWD, TDI
2017	Stevanovic et al	54.9 ± 7.3/52.6 ± 5.2	162	GLS	E/A, EDT, E/Em	2D STE, TDI
2017	Ringle et al	37.6 ± 9/35.1 ± 7	92	GLS, GCS, GRS	SrE, E/SrE	2D,3D STE, PWD, TDI
2017	Natali et al	≥ 40 and ≤ 80	75	GLS	E/E', SrE	2D STE, PWD, TDI
2017	Almorós et al	Review	-	GLS, GCS, GRS	E/A, E/E'	2D STE, PWD, TDI
2017	Derumeaux	Editorial comment	-	GLS	E/E'	2D STE, TDI
2017	Mochizuki et al	55 ± 17/56 ± 16/57 ± 17	259	GLS	E/A, EDT, E/E'	2D STE, PWD, TDI
2017	Aboukhoudir et al	84- Case Report	-	GLS	E/E'	2D STE,
2017	Ernande et al	57.4 ± 9.1	745	GLS	E/A, EDT, E/E'	2D STE, PWD, TDI
2017	Vasanji et al	52 ± 6/51 ± 6	28	GLS, GCS, GRS	-	CMR
2017	Tadic et al	49 ± 1.3/54 ± 1	104	GLS, Multilayer	E/E', E/E'	2D STE, TDI
2017	Armstrong et al	<50 yo – 390 >=50 yo- 324	714	GCS	-	CMR
2017	Karagodin et al	68.6 ± 9.9/68.5 ± 9.9	154	-	E/A, EDT, E/E', DWS	2D STE, PWD, TDI
2017	Leung et al	Not mentioned	50	GLS	E/A, EDT, E/E'	2D STE, PWD, TDI
2016	Loncarevic et al	54.8 ± 4.9/54.8 ± 7.7/54.6 ± 5.8/55.3 ± 5.4	290	GLS, GRS	E/A, EDT, E/E'	2DSTE, PWD, TDI
2016	Ismail et al	63.2 ± 8.9/61.1 ± 10.6	218	Atrial longitudinal strain, Peak atrial contraction strain	E/A, EDT, E/E', PALS, PACS	2D STE, PWD, TDI

(Continues)

TABLE 1 (Continued)

Year	Study	Age	No of patients	Subclinical Systolic Dysfunction	Diastolic Dysfunction	Technique
2016	Enomoto et al	52 ± 16/56 ± 15	112	GRS, GLS, GCS	E/A, EDT, E/E'	2D STE, PWD, TDI
2016	Mochizuki et al	52 ± 16 55 ± 15 58 ± 16	267	GRS, GLS, GCS	E/A, EDT, E/E'	2D STE, PWD, TDI
2016	Bjornstad et al	15.0 ± 1.9 15.1 ± 2.1	89	GLS	E/E'	2D STE, PWD, TDI
2016	Jensen et al	49.5	1075	GLS	E/E'	2D STE, PWD, TDI
2016	Nouhravesh et al	49.6	1090	GLS, GCS, GRS	E/A, EDT, E/E', Et, At	2D STE, PWD, TDI
2016	Bonapace et al	68.8 ± 6.2/68.9 ± 5.1	180	GLS	SrE, SrL, E/SrE	2D STE
2016	Chang et al	52.2 ± 8.5/55.3 ± 8.8	25	GLS, GCS	E/A, EDT, E/E'	2D STE, PWD, TDI
2016	Qiao et al	Rabbits	36	GLS, GCS, GRS	LVIDd, LVIDs	2D STE, PWD, TDI
2016	Htike et al	18-50	90	Not specified	PEDSR	2D STE
2016	Levelt et al	50 ± 10/56 ± 9/56 ± 8	54	Peak circumferencial strain	Peak CSR	CMR
2016	Chen et al	56 ± 4	20	GLS, GRS	GLSR, GRSR	CMR
2016	Hodzic et al	12.4 ± 3.4/12.8 ± 2	60	GLS	E/A, EDT, E/E'	2D STE, PWD, TDI
2016	Jeong et al	Mice	-	GCS	CSR	CMR
2016	Leung et al	54 ± 10	105	GLS	E/A, EDT, E/E'	2D STE, PWD, TDI
2016	Liu et al	Rats	36	GRS, GCS	E/A, EDT, E/E'	2D STE, PWD, TDI
2015	Zhen et al	59.2 ± 9.7	108	GLS	E/A, EDT, E/E'	2D STE, PWD, TDI
2015	Jedrzejewska et al	30.7 + 7.2/27.3 + 4.9	100	GLS, GCS, GRS	E/A, EDT, E/E'	2D STE, PWD, TDI
2015	Leung et al	56 ± 7 years,	8	GLS	E/A, EDT, E/E'	2D STE, PWD, TDI
2015	McCormick et al	64.8 ± 7.0/66.1 ± 4.6	22	GLS	E/A, EDT, E/E'	2D STE, PWD, TDI

Abbreviations: 2D STE, two-dimensional speckle-tracking echocardiography; 3D-STE, three dimensional speckle-tracking echocardiography; CACS, coronary calcium score; CMR, Cardiac magnetic resonance; CSR, circumferential strain rate; CT, computerized tomography; E/A, Early to late diastolic transmitral flow velocity; E/E', Transmitral early filling velocity to early diastolic mitral annular velocity; Ecc, circumferential peak systolic strain; EDT, E wave decelaration time; Ell, longitudinal peak systolic strain; tEcc, tEll, time to peak strain; GAS, global area of strain; GAVS, global atrioventricular strain; GCS, Global circumferential strain; GCSR-E, global circumferential early peak diastolic strain rate; GLPSS, Global peak longitudinal strain; GLS, Global longitudinal strain; GLSR-E, global longitudinal early peak diastolic strain rate; GRS, Global radial strain; GSrL, global peak diastolic longitudinal strain rate; LACS, left atrium global circumferential strain; LALS, left atrium global longitudinal strain; LARS, left atrium global radial strain; LAScd, left atrial longitudinal strain during conduit phase; LASct, left atrial longitudinal strain during contraction phase; LASr, left atrial longitudinal strain during reservoir phase; LSR, Longitudinal strain rate; LVCS, left ventricular global circumferential strain; LVLS, left ventricular global longitudinal strain; LVRS, left ventricular global radial strain; PALS, peak atrial longitudinal strain; PEDSR, peak diastolic strain rate; pLASRcd, peak left atrial longitudinal strain rate during conduit phase; pLASRct, peak left atrial longitudinal strain rate during contraction phase; pLASRr, peak left atrial longitudinal strain rate during reservoir phase; PSSR, peak systolic strain rate; PWD, Pulsed-wave Doppler; RSR, radial strain rate; RVLS, right ventricular global longitudinal strain; SrE, Strain rate at early diastole; SrIVR, Strain rate at isovolumic relaxation; TDI, Tissue Doppler imaging; TG, triglycerides.

Diastolic dysfunction was measured in most of the studies included either by tissue Doppler imaging E' and E/E' ratio, or by conventional transthoracic echocardiographic parameters: transmitral E/A ratio and deceleration time[2,3](Table 1). However, there are several studies that use STE for diastolic dysfunction assessment, measuring the peak strain rate during early diastole and the ratio between E wave and the peak strain rate during early diastole.[2,4,7] Diastolic dysfunction is correlated with lower E/A ratio values and higher E/E' ratio values by Doppler imaging,[3] and with a lower peak longitudinal strain rate in early diastole[2,4] and a higher ratio between E wave and the peak strain rate in early diastole by STE.[7]

Subclinical systolic dysfunction was predominantly assessed using 2D STE parameters: GLS was measured in most of the cases, but global circumferential (GCS) and radial (GRS) strains were also measured in some of the included studies[4,7,8,16,21-25](Table 1). Furthermore, few studies also used 3D STE[1,7,26] and multiple-layer strain[5,8,21,27-30] for systolic function assessment. Systolic dysfunction was correlated with lower absolute values of GLS, GCS or GRS,[4,7] GLS being independently associated with cardiovascular events, short- and long-term hospital mortality, all-cause death and hospitalization.[1,5,14,17,18,21,31-33] Also, the association between GLS and dyspnoea in DM patients with preserved LV ejection fraction[34] brings further proof that GLS is

a better predictor of adverse cardiovascular effects compared to LV ejection fraction in DCM patients.[7,21,35] The cut-off values of all 3 systolic dysfunction parameters are showed in Table 3.

Diastolic dysfunction is traditionally believed to be the hallmark of preclinical DCM, most of the literature studies arguing that it is the first disease manifestation.[1-3,10,14,15,36-40] Lorenzo-Almoros et al support the theory that diastolic dysfunction is already established in the initial stages of DCM.[1] Similar results were found in 2 animal studies which further characterized the progression of myocardial dysfunction from diastolic to systolic, and finally to overt HF.[10,15] Moreover, Htike et al and Georgievska-Ismail et al also reported the presence of diastolic dysfunction from the earliest stages of DCM,[39,40] the latter suggesting that diastolic dysfunction is independently associated with DM, regardless of the presence of systolic dysfunction.[40] Also, Mochizuki et al demonstrated that the development of systolic dysfunction in DM patients with preexisting diastolic dysfunction accelerates the progression of the latter.[3]

The last years have brought solid evidence with regard to the progression of myocardial dysfunction in patients with DM, several studies showing that diastolic dysfunction might not be the earliest alteration in DCM.[5,8,16,17,41] To that end, a breakthrough was the finding that patients with DM presenting diastolic dysfunction as the earliest sign of DCM were more likely to present other associated cardiovascular risk factors, such as older age, obesity or higher heart rates.[14,38,41,42] However, there are also studies reporting an association between cardiovascular risk factors and systolic dysfunction.[3,5,43] According to Ringle et al, this can be explained by the fact that most studies included patients with significant underlying cardiovascular risk factors, which may also play an important role in the development of myocardial dysfunction. The authors evaluated young type 1 DM patients without additional cardiovascular risk factors, in which they observed that both systolic and diastolic myocardial functions were impaired in early stages of DCM.[7] The presence of concomitant systolic and diastolic dysfunctions in early stages of DCM was also indicated by several other studies in both type 1 and type 2 DM patients.[4,11,18,24,26]

Nevertheless, Ernande et al were the first to call into question diastolic dysfunction as being the first marker of DCM and to highlight the fact that systolic function may be impaired despite preserved diastolic function. Their results showed that even though diastolic dysfunction was present in 47% of the DM patients included, 28% had systolic dysfunction despite normal diastolic function. In this respect, the authors concluded that systolic dysfunction assessed by systolic strain alteration 'might be proposed as the first indicator of DCM'.[41] Several other studies found similar results in both type 1 and type 2 DM patients, which further acknowledge the presence of systolic dysfunction in early stages of DCM.[5,8,16,17]

GLS was the preferred parameter for systolic function assessment and, at the same time, the most often impaired, followed by GCS and GRS, in that order. A possible explanation for this may be that in the early stages of DCM, the longitudinally oriented subendocardial fibres are the most vulnerable, being first affected by the pathological process.[5,8,21,28] As a defence mechanism, the circumferential and radial fibres from the mid-myocardial and subepicardial layers increase their function, thus the only STE parameter affected being GLS. In this stage, the subclinical form of DCM is present, which manifests as HFpEF. With the progression of the disease, the mid-myocardial and epicardial layers are also affected, DCM evolving to its clinical stage, HF with reduced ejection fraction.[8,19] Further clinical evidence in this direction was brought by Brane Loncarevic et al, who reported GLS impairment in all DM patients included in their study, independently of the presence of symptoms, hypertension or coronary artery disease, while GCS impairment was only found when DM was associated with either hypertension or coronary artery disease.[4] Another possible cause for the more frequent use of GLS can be its recent validation as a quantitative index for LV function.[12]

3.3.2 | Left atrial and right ventricular myocardial dysfunction assessment

DM is considered one of the principal causes of left atrial remodelling, increasing the risk of HFpEF and atrial fibrillation.[19,44] STE strain measurement allows quantifying left atrial function even before atrial structural remodeling.[44,45] L. Georgievska-Ismail et al investigated the role of STE in the assessment of left atrial systolic dysfunction in DM patients and reported lower GLS values, concluding that 'left atrial deformation mechanics are impaired in patients with DM and HFpEF'.[40] Also, Y. Mochizuki et al demonstrated concomitant left atrial systolic and diastolic dysfunctions in type 2 DM patients with diabetic nephropathy and albuminuria.[44] Furthermore, in the investigation of S. Bonapace et al, the incidence of atrial fibrillation after a 2-year follow-up was higher in DM patients than in controls. The authors showed that early systolic dysfunction is one of the first myocardial alterations predisposing to new-onset atrial fibrillation, which might be partly due to preexisting DM complications: coronary microvascular dysfunction, myocardial perfusion defects or increased myocardial fibrosis.[19]

In a study by Cameli et al, peak left atrial longitudinal strain and global atrial-ventricular strain were better predictors of subclinical myocardial dysfunction compared to GLS in asymptomatic patients with hypertension and DM.[46] Furthermore, Mohseni-Badalabadi et al showed that left atrium longitudinal strain was reduced in diabetic obese patients.[47]

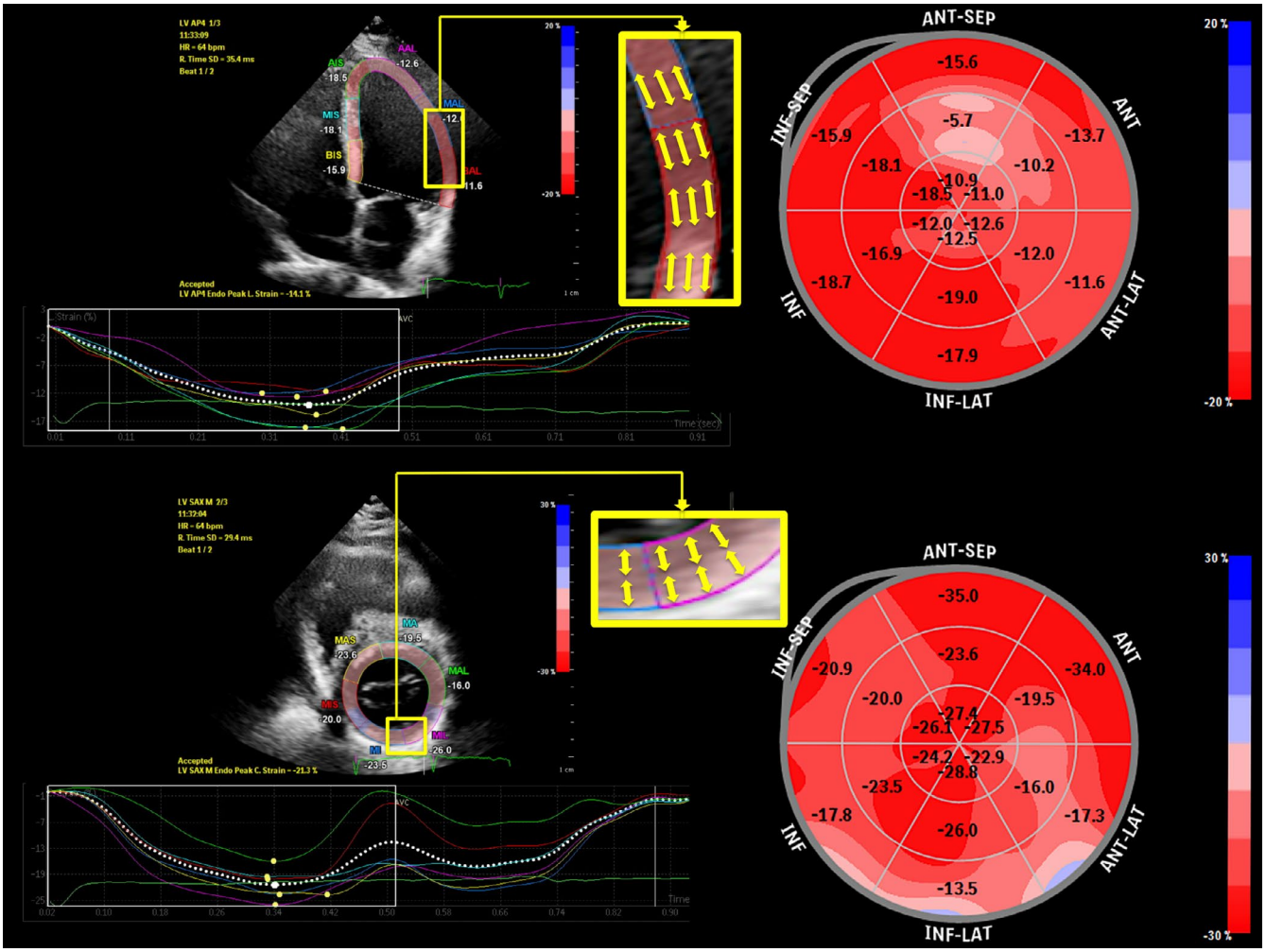

FIGURE 2 Speckle-tracking echocardiographic analysis of myocardial deformation. Measurement of regional (A) longitudinal strain and (B) circumferential strain, with corresponding bullseye maps of global longitudinal and circumferential strain

Several studies identified right ventricular systolic and diastolic dysfunctions in both type 1 and type 2 DM patients,[8,27,48] right ventricular global and layer-specific strain being identified as an important morbidity and mortality predictor.[27] Type 1 DM was associated with both LV and right ventricular systolic dysfunction, and more discrete diastolic dysfunction, which brings further proof for the early systolic dysfunction presence in DCM.[8,48]

3.3.3 | Young adults and children

Young adults suffering from type 2 DM are more prone to develop premature atherosclerotic changes and cardiovascular diseases and to present early subclinical diastolic and systolic dysfunctions.[39] Also, 2 studies showed that children and adolescents with type 1 DM may present impaired systolic function measured by GLS despite normal diastolic function.[35,49] Ernande et al further demonstrated this relation in type 2 DM patients, finding that young men without associated arterial hypertension or obesity are more often identified with subclinical systolic dysfunction and LV hypertrophy and have the worst outcomes, compared to elderly, obese and hypertensive women which are more often associated with diastolic dysfunction and better outcomes.[14] A more recent study observed systolic dysfunction among type 1 DM late-teens being proportional to DM duration.[30]

3.3.4 | Association between microvascular complications of diabetes mellitus and myocardial dysfunction

Two studies investigated the link between arterial stiffness and myocardial dysfunction. Theliade et al reported that aortic and peripheral arterial stiffness predispose to both early systolic and diastolic dysfunction in type 1 DM patients,[20] while Karagodin et al found that proximal aortic stiffness leads to diastolic dysfunction and HFpEF. However, the

TABLE 2 Comparison of STE with other imaging techniques. Advantages and limitations[1,8,13,21,79,80]

	Advantages	Limitations
TDI	• High availability • Direct calculation of strain rate • Good reproducibility • Excellent temporal resolution (better for high heart rate) • Objective quantitative evaluation of local deformation • Online measurements of velocities (spectral pulsed tissue Doppler imaging)	• Assessment of limited regions of tissue • Cannot discriminate passive motion (related to translation or tethering) from active motion (fiber shortening or lengthening) • Angle dependency • Need of high frame rates • Intra- and interobserver variability • The need of fixed sample volume • Influenced by global heart motion • Noise interference
STE	• Assessment of global myocardial function • Angle independency • Study of myocardial segments motion in any direction *longitudinal, circumferential and radial strains) • High reproducibility • Wide clinical application • Less noise interference and artefacts	• Dependent on good image quality • Need of high frame rates • Intervendor variability • Lack of definite reference ranges • Lack of standard instructions for measuring it adequately • Relative low availability
CMR	• Better in patients with suboptimal echocardiographic image quality • Superior myocardial volumetric and functional assessment • Golden standard for right ventricle function • Superior for complex myocardial geometry • High reproducibility	• High cost • Low availability • Time consuming • Challenging or impossible in patients with claustrophobia or implanted metal devices • Lack of validation • Manual tracing of the endo- and epicardial borders and definition of end-diastole based on visual assessment of LV dimensions • Cannot be used interchangeably with STE for control

Abbreviations: CMR, cardiac magnetic resonance; STE, speckle-tracking imaging; TDI: tissue Doppler imaging.

TABLE 3 Cut-off values for principal systolic STE parameters used—GLS, GCS and GRS[70,73,81]

Right ventricle	No definite reference ranges are currently recommended for either global or regional RV strains	>−20 (<20 in magnitude with the negative sign)—likely abnormal.
Left ventricle Reference values (lower limit of normality)[1]	• GLS	In the range of −20%—healthy person; the lower the absolute value of strain is below this value, the more likely it is to be abnormal -17% to −21%
	• GCS	-17% to −20%
	• GRS	47% to 59%

Abbreviations: GCS, global circumferential strain; GLS, global longitudinal strain; GRS, global radial strain.

[1]Heart failure risk increases the lower the absolute value of strain is below these values.

latter did not investigate subclinical systolic function parameters in its group.[50] Also, DCM evaluated by 2D-STE has been shown to precede diabetic polyneuropathy in a study conducted by Tabako et al, both systolic and diastolic functions being impaired.[22]

Moreover, N. Nouhravesh et al showed that in type 1 DM patients with already developed microvascular complications such as diabetic retinopathy, both systolic and diastolic functions were impaired.[4,51]

3.4 | Further assessment of diabetic cardiomyopathy

Three-dimensional STE is a new, less reproducible echocardiographic method, with higher specificity for myocardial dysfunction. M. Enomoto et al emphasized the advantages of 3D STE in their study on the relationship between myocardial dysfunction and diabetic microangiopathy: it is not affected by cut-down views and avoids any potential out-of-plane

motion of the ultrasonic speckles. The results showed a strong correlation between early longitudinal systolic dysfunction measured by GLS and autonomic neuropathy of the myocardium.[26] Chen et al also highlighted the importance of 3D STE for the assessment of subclinical systolic dysfunction in type 2 DM patients with poor glycaemic control.[25]

Moreover, a new STE marker – peak systolic longitudinal rotation was found to be impaired in early stages of DCM showing promising perspectives for the future.[52]

In the last 3 years, several studies have investigated myocardial strain using cardiac magnetic resonance (CMR). The results showed similar associations between DCM and myocardial dysfunction as STE, but with higher costs. These studies go beyond the purpose of our review.[7,37,38,43,53-63] The principal advantages and limitations of CMR compared to STE and TDI are showed in Table 2.

Furthermore, A. Lorenzo-Almoros et al reported specific plasma biomarkers that can be useful, alongside STE, in the investigation of myocardial dysfunction in DCM patients.[1] Of these, it is important to mention NT-proBNP, multiple studies showing a better association between GLS and NT-proBNP than between GLS and LV ejection fraction in assessing myocardial systolic function in these patients.[1,21]

3.5 | Factors that may improve myocardial function in DM patients

Over the past years, several studies have investigated the role that external factors such as intensive glycaemic control, weight loss or certain drugs may have on DCM progression or remission.

Intensive glycaemic control in DCM patients is a controversial subject. S. Costantino et al demonstrated that intensive glycaemic control measured by glycated haemoglobin (HbA1c < 6.5%) in mice does not lower the burden of LV dysfunction.[9] Furthermore, Lorenzo-Almoros et al showed that intensive glycaemic control in humans (HbA1c < 7%) does not prevent cardiovascular complications in patients suffering from DM,[1] and several other studies found no association between HbA1c levels and systolic dysfunction.[8,64] However, many recent studies found that the biggest improvements in diastolic and systolic functions were seen in patients with the largest reduction in HbA1c levels.[2,5,18,25,33,43,65]

Several studies outline the importance of weight loss in the reversibility of systolic and diastolic dysfunctions in DM patients.[42,65,66] M. Leung et al found that weight loss in type 2 DM was as important as glycaemic control for the improvement of systolic and diastolic function, the improvement being even greater when both glycaemic control and weight loss were obtained.[65] The same team demonstrated in another study that patients who develop worsened glycaemic control, even after a long period of proper glycaemic control, experience further deterioration of LV systolic function.[18] Another study found that obesity predisposed to both systolic and diastolic dysfunctions.[42] Albu et al emphasized the importance of physical exercise in the management of the cardiovascular risk factors in DM subjects and its beneficial effect on the left ventricular diastolic dysfunction. The experimental and clinical research revealed encouraging results physical activity might slow the development of CMD or even regress the changes associated with it.[67]

The association between weight loss, glycaemic control and LV myocardial function improvement was once again confirmed by M. Leung et al in a study on the effects of sleeve gastrectomy on the already mentioned parameters. The results showed a significant improvement in both diastolic and systolic function after surgery, the principal associated factor being glycaemic control improvement.[64]

Apart from that, certain drugs have demonstrated a beneficial role in slowing down DCM progression. Sacubitril-valsartan showed better glycaemic control in patients with HF and DM.[2] Another renin-angiotensin-aldosterone system inhibitor, telmisartan, showed an increase in systolic function assessed by GLS and GCS,[68] while liraglutide, a GLP-1 agonist, ameliorated diastolic dysfunction in DCM patients. Also, metformin[18] was demonstrated to ameliorate systolic dysfunction and to lower morbidity and mortality in DCM patients In addition, sodium-glucose-lipid-transporters such as empagliflozin and dapagliflozin have shown a reduction in cardiovascular mortality in patients with DCM, the latter being recently approved for HFrEF treatment, regardless of DM presence.[6,9,18,69]

All these studies together highlight the importance that both intensive glycaemic control and weight loss have and that some drugs may have in the management of DCM and in preventing its progression from subclinical to overt stages.

3.6 | The role of STE in current guidelines

The value of speckle tracking in quantifying left ventricular function has been recognized by the current guidelines ASE/EACVI 2015 and ACC/AATS/AHA/ASE/ASNC/HRS/SCAI/SCCT/SCMR/STS 2019 and EACVI/ASE/Industry Task Force 2018.[70-72] Its application in routine clinical echocardiography for all chambers is recommended in centres which have appropriate platforms and experience.[70-72] Even though GLS resulting from speckle-tracking echocardiography represents a practical and reproducible approach in terms of analysing the function of the left ventricle for clinical practice in numerous heart pathologies, caution must be payed taking into consideration the interobserver variability together with the different software versions currently available.[70]

3.7 | Gaps in knowledge and future perspectives

Although significant advances have been made in these past years for the assessment of subclinical myocardial dysfunction in DCM, there are still some gaps in achieving and optimizing better diagnosis and prevention for these patients.

A primary challenge comes from the lack of strong, evidence-based studies of myocardial function assessment using STE in DM patients. Although STE is an established method for myocardial function assessment, larger randomized studies are necessary in order to clearly demonstrate the progression of myocardial dysfunction in DCM patients.[3,4] Another major challenge comes from STE limitations. Its dependence on good image quality, intervendor variability, the lack of definite reference ranges and standard instructions for measuring it adequately, and its relative low availability worldwide may postpone standardizing protocols and replacement of traditional echocardiographic parameters such as left ventricle ejection fraction.[3,70]

However, as technology advances and the use of STE is continuously increasing in both clinical and research fields, further diagnosis and prognosis insights continue to emerge.[73,74] Even though GLS is the most thoroughly studied STE parameter and GLS-guided management of DM patients showed good results in the prevention of DCM progression to later stages,[75] other strain parameters also showed promising results.[76] LA STE parameters showed better sensitivity compared to traditional indices of atrial function in various heart disease.[77] Also, both 2D and 3D right ventricle STE parameters showed encouraging value as an alternative to current standard—CMR, for the evaluation of subclinical myocardial dysfunction.[78] As DCM is known to remain asymptomatic for many years before progressing to overt HF,[1] there is substantial evidence that STE will become standard for the evaluation of myocardial dysfunction in this group of patients in the near future.[73,74]

4 | CONCLUSIONS

Diabetic cardiomyopathy has evolved over the last decades, becoming a real pathology with life-changing consequences if left unassessed and untreated. Although LV diastolic dysfunction is traditionally believed to be the first myocardial alteration in DCM, systolic dysfunction has been recently shown to be present in early DCM stages. STE proved to be a useful technique in diagnosing subclinical systolic dysfunction in DCM patients, with or without associated diastolic dysfunction. This could be essential in understanding the interconnection between HF and DM and shows promise for developments in early management of DCM.

ACKNOWLEDGEMENTS

Special thanks to Denisa Marineanu for the language editing service.

CONFLICT OF INTEREST

No competing financial interests exist.

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How to cite this article: Minciună I-A, Hilda Orășan O, Minciună I, et al. Assessment of subclinical diabetic cardiomyopathy by speckle-tracking imaging. *Eur J Clin Invest*. 2021;00:e13475. https://doi.org/10.1111/eci.13475

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2021, Article ID 8671713, 15 pages
https://doi.org/10.1155/2021/8671713

Review Article

Endothelial Dysfunction, Inflammation, and Oxidative Stress in COVID-19—Mechanisms and Therapeutic Targets

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Received 26 June 2021; Accepted 7 August 2021; Published 25 August 2021

Academic Editor: Vladimir Jakovljevic

The outbreak of the COVID-19 pandemic represents an ongoing healthcare emergency responsible for more than 3.4 million deaths worldwide. COVID-19 is the disease caused by SARS-CoV-2, a virus that targets not only the lungs but also the cardiovascular system. COVID-19 can manifest with a wide range of clinical manifestations, from mild symptoms to severe forms of the disease, characterized by respiratory failure due to severe alveolar damage. Several studies investigated the underlying mechanisms of the severe lung damage associated with SARS-CoV-2 infection and revealed that the respiratory failure associated with COVID-19 is the consequence not only of acute respiratory distress syndrome but also of macro- and microvascular involvement. New observations show that COVID-19 is an endothelial disease, and the consequent endotheliopathy is responsible for inflammation, cytokine storm, oxidative stress, and coagulopathy. In this review, we show the central role of endothelial dysfunction, inflammation, and oxidative stress in the COVID-19 pathogenesis and present the therapeutic targets deriving from this endotheliopathy.

1. Introduction

The SARS-CoV-2 virus, responsible for COVID-19 disease, can evolve with a wide range of clinical manifestations, from mild forms manifesting as fever, dyspnea, cough, and loss of smell and taste to severe forms, especially in the elderly with comorbidities, characterized by respiratory failure due to severe alveolar damage [1]. In the extremely severe forms of the disease, rapidly progressive multiple organ failure occurs, which manifests through complications such as shock, acute cardiac injury, acute respiratory distress syndrome (ARDS), disseminated intravascular coagulopathy (DIC), and acute kidney injury, which may ultimately prove fatal [2]. Recent studies have demonstrated that respiratory failure occurring in COVID-19 is due not only to acute respiratory distress syndrome but also to macro- and

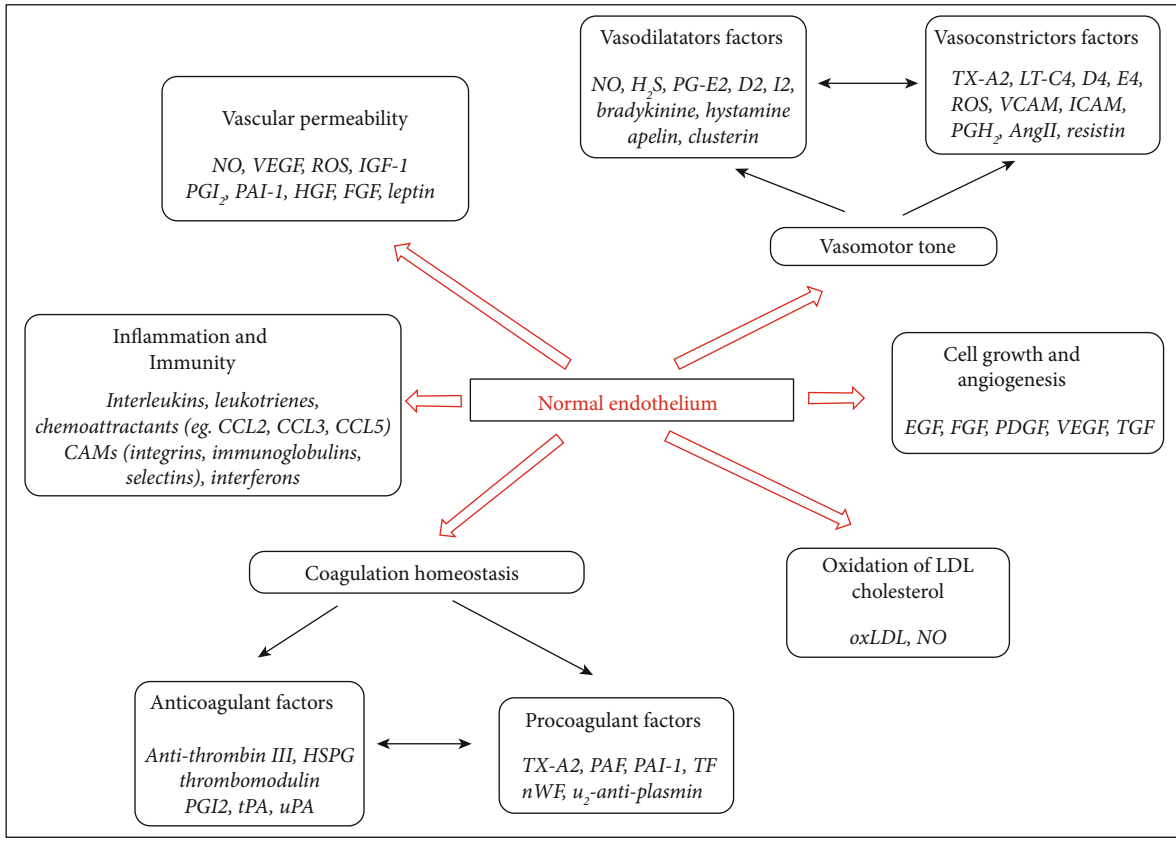

Figure 1: Functions of vascular endothelium. Endothelium cells produced some vascular mediators/factors that accomplished the six major functions of normal endothelium (modulation of vascular permeability and vasomotor tone modulation, coagulation homeostasis, inflammation and immunity regulation, cell growth regulation, and oxidation of LDL cholesterol) by which the vascular homeostasis is maintained (adapted after [9]).

microvascular involvement [3–5], a particular role being played by vascular endothelial damage [6, 7]. New observations show that COVID-19 is an endothelial disease [8] and that endotheliopathy is responsible for inflammation, cytokine storm, oxidative stress, and coagulopathy. An argument of this theory is the fact that patients who have endothelial dysfunction due to various comorbidities (obesity, hypertension, and diabetes) develop more severe forms of COVID-19, explained by an additional alteration of the already dysfunctional vascular endothelium [7].

In this review, we show the central role of endothelial dysfunction, inflammation, and oxidative stress in the development of complications of SARS-CoV-2 infection and their pathophysiological consequences, and examine the main therapeutic targets deriving from this endotheliopathy.

The endothelium, one of the largest organs of the human body, is capable of producing a wide variety of molecules, with effects that are often contradictory, with a role in maintaining homeostasis, such as vasodilator and vasoconstrictor, procoagulant and anticoagulant, inflammatory and anti-inflammatory, fibrinolytic and antifibrinolytic, and oxidant and antioxidant substances [9].

The normal endothelium regulates vascular homeostasis through six major functions: (1) modulation of vascular permeability, (2) modulation of vasomotor tone, (3) modulation of coagulation homeostasis, (4) regulation of inflammation and immunity, (5) regulation of cell growth, and (6) oxidation of LDL cholesterol (Figure 1). These functions are achieved through numerous mediators, of which the most studied is nitric oxide (NO) [9].

Nitric oxide is the most important vasodilator substance produced by endothelial cells. NO also has an antithrombotic action, inhibiting the fibrotic properties of angiotensin II and endothelin I by downregulating the receptors for these molecules. NO is synthesized in endothelial cells from L-arginin under the action of the endothelial NO synthase (eNOS) [10]. This reaction requires the presence of molecular oxygen and certain cofactors, including calmodulin, tetrahydrobiopterin (THB4), NADPH (adenine dinucleotide phosphate), flavin adenine dinucleotide, and flavin mononucleotide. From this reaction, L-citrulline as a by-product results [11].

Endothelial dysfunction is defined as a reduction in the bioavailability of vasodilator substances, especially NO, and an increase in vasoconstrictor substances.

The reduction of NO bioavailability can be due to a decrease in eNOS production (lack of cofactors necessary for eNOS synthesis) on the one hand, and to an increase in excessive NO degradation or inactivation by reactive oxygen species (ROS), on the other hand [12]. The increase in the

production of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\bullet$), hypochlorous acid (HOCl), and lipid superoxide radical, represents the main cause of the decrease in NO bioavailability in cardiovascular diseases [13]. Under physiological conditions, ROS production is controlled by an effective system of antioxidants, molecules that are capable of neutralizing ROS, thus preventing oxidative stress. In tissues, natural enzymatic antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase, and catalase, play an important role in the conversion of ROS to oxygen and water. In pathological conditions, ROS can be present in excess relatively to the existing antioxidant capacity. This alteration of the balance in favor of oxidation termed "oxidative stress" may have negative effects on cell and tissue function [9].

Endothelial cells (EC) possess a number of mechanisms that reduce local oxidative stress. When subjected to shear stress, the endothelium produces SOD, which eliminates ROS [14]. The endothelial cell can also express glutathione peroxidase, which can mitigate oxidative stress [15]. Similarly, haem-oxygenase provides another mechanism by which the endothelial cell can resist to local oxidative stress [16, 17].

In contrast, proinflammatory cytokines can stimulate endothelial cells to mobilize NADPH-oxidase that generates superoxide anions, amplifying local oxidative stress [18, 19].

2. COVID-19-Associated Endotheliopathy and Oxidative Stress

Endothelial dysfunction or endotheliopathy is an important pathological characteristic in COVID-19 [20]. Electron microscopy of blood vessels in autopsy samples from patients with COVID-19 revealed the presence of endothelial cell degradation and apoptosis [21, 22]. Endothelial dysfunction biomarkers, such as thrombomodulin, von Willebrand factor (vWF), angiopoietin 2, and PAI-1, are frequently increased in patients with COVID-19 compared to healthy persons and seem to have prognostic significance, being associated with more severe forms of the disease and high mortality [23, 24]. Endothelial dysfunction is an important factor in the pathophysiology of thrombotic complications associated with COVID-19, including myocardial infarction and stroke [23, 24].

At present, it is uncertain whether endotheliopathy associated with COVID-19 is the result of direct endothelial cell viral infection, as reported in some autopsy studies [21, 25] or is a consequence of the inflammatory response induced by the virus.

Many pathophysiological mechanisms have been described which explain the implication of endothelial dysfunction in the occurrence of microvascular involvement in COVID-19 infection. Microvascular cerebral involvement in COVID-19 as a result of age-related endothelial dysfunction is an important challenge for research [20]. Overactivation of poly-(ADP-ribose) polymerase 1, as can be observed in viral infections, can lead to NAD+ depletion and subsequent endothelial dysfunction [26, 27]. In addition, the dysfunction of the nuclear factor erythroid 2-related factor 2 (NRF2) antioxidant defense pathway in endothelial cells might also play a role in the COVID-19 associated endotheliopathy [28]. The pharmacological activators of NRF2 were proposed as potential treatment options for COVID-19 [29]. NRF2 has strong anti-inflammatory and antiapoptotic effects in endothelial cells. It should be noted that NRF2 dysfunction exacerbates the deleterious effect of hypertension and diabetes on the endothelium, conditions known for the increase in the COVID-19-related risk of death [29].

Oxidative stress is generated by high Ang II concentrations and low Ang 1-7 concentrations (Figure 2). These ROS can oxidize cysteine residues in the peptidase domain of receptors ACE2 and RBD of proteins SARS-CoV and SARS-CoV-2, maintaining them in oxidized forms (disulfide), unlike reduced forms (thiol) [30]. It is possible that oxidation of these thiols to disulfides, through an oxidative stress mechanism, may increase the affinity of proteins SARS-CoV and SARS-CoV-2 S for ACE2 receptors and, consequently, increase the severity of COVID-19 infection [31].

The relationship between Ang II and NADPH-oxidase was investigated using murine smooth vascular muscle cells. When the cells were exposed to Ang II, the researchers observed an increased activity of NADPH-oxidase, as well as an increased production of superoxide anions. The exact mechanisms for the stimulation of NADPH-oxidase are complex, genetically mediated, at transcriptional and post-transcriptional level, and involve numerous signaling molecules and scaffolding proteins/platforms [32]. Inactive NADPH-oxidase contains two subunits: glycoprotein (gp) 91phox and p22phox. In the presence of Ang II, NADPH-oxidase is activated through the involvement of additional subunits p67phox, p47phox, p40phox, and Rac1. Activated NADPH-oxidase can generate superoxide anions. Studies in mice have shown that increased NADPH-oxidase activity can be found even in the absence of ACE2 [33, 34]. Since binding of SARS-CoV-2 to ACE2 receptor inhibits the catalytic activity of the enzyme, i.e., the conversion of Ang II to Ang 1-7, the activity of NADPH-oxidase increases in patients with SARS-CoV-2, subsequently leading to an increase in oxidative stress [35].

In a recently published study [36], the long-term effects of SARS-CoV-2 virus on oxidative stress and vascular endothelium were discussed. Thus, it was proposed that SARS-CoV-2, by inducing mitochondrial dysfunction and oxidative stress, can initiate a feedback loop promoting a chronic state of inflammation and endothelial dysfunction even after the viral particles have been eliminated from the body. In this proposed mechanism, SARS-CoV-2 first induces activation of NADPH-oxidase, which produces superoxide (O_2^-), a ROS that is involved in reactions which deteriorate the electron transport chain (ETC) [32, 37].

Increased oxidative stress and inflammation resulting from this mitochondrial dysfunction subsequently initiate a feedback loop that perpetuates NADPH-oxidase activation, mitochondrial dysfunction, inflammatory cytokine production and loss of identity of EC [36]. Considering these hypothetical long-term consequences of SARS-CoV-2 infection on blood vessels, the treatment of chronic oxidative stress

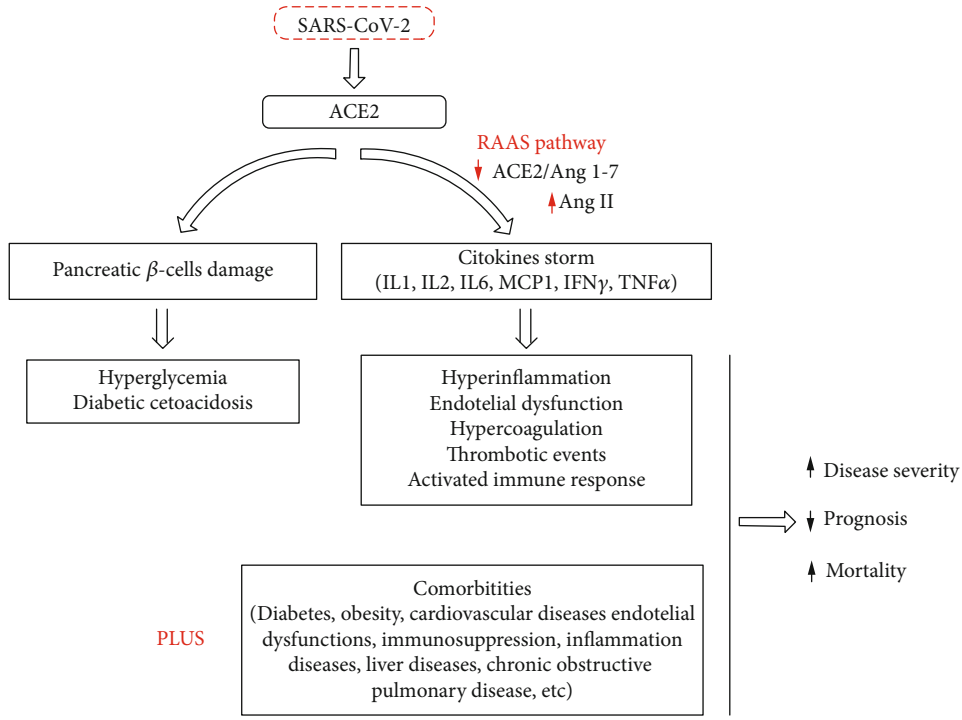

Figure 2: SARS-CoV-2 enters the human body by binding to ACE2. Activation of RAAS produced a cytokine storm, resulting in the secretion of proinflammatory cytokines/chemokines such as interleukins (ILs), interferon-gamma (IFN-γ), monocyte chemoattractant protein-1 (MCP1), and tumor necrosis factor-alpha (TNF-α). This storm produces a pleiades of phenomena which is associated with preexistent comorbidities that lead to an increase in disease severity (adapted after [31]).

and inflammation in EC can be essential in preventing future complications among millions of persons currently diagnosed with COVID-19 [38].

3. COVID-19 Endotheliitis

Numerous postmortem histopathological examinations in patients who died of COVID-19 not only revealed the presence of endotheliitis in the key organs affected by SARS-CoV-2, but also demonstrated the presence of viral structures within the endothelial cells by electron microscopy [21, 25, 39, 40]. By analyzing samples from the transplanted kidney in a COVID-19 patient who developed multiorgan failure, Varga et al. [25] demonstrated the capacity of the virus to invade endothelial cells. In the same patient, histological findings showed the inflammatory infiltrate of the endothelium and the morphological changes that occur during apoptosis in the heart, small bowel, and lungs. Furthermore, they proved the presence of endotheliitis in the lung, heart, kidney, liver, and small intestine of two other COVID-19 patients by postmortem analysis [25]. The wide distribution of ACE2 receptor in endothelial cells explains the multiorgan affinity of the virus, confirmed once more in a study by Puelles et al. The presence of viral particles in the pharynx, lungs, heart, blood, liver, kidneys, and brain was established despite the level of viral load [39].

The electron microscopy studies performed by Ackermann et al. [21] proved the presence of SARS-CoV-2 within the endothelial cells and in the extracellular space; furthermore, ultrastructural injury of the endothelium was also present. The authors of the aforementioned study compared the histological changes that occur in the lungs of SARS-CoV-2 patients with those occurring in acute respiratory distress syndrome caused by influenza A (H1N1) and ten uninfected control lungs. The results revealed that the lungs of COVID-19 patients presented disseminated alveolar injury associated with necrosis, lymphocytic inflammation, and microthrombosis. In addition, the expression of angiotensin-converting enzyme 2 (ACE2) investigated by immunohistochemical analysis was present in lymphocytes only in the COVID-19 and influenza groups [21].

The postmortem electron microscopy analysis of the kidney tissue of 26 patients with COVID-19 from China revealed the presence of coronavirus-like particles in the renal tissue. Furthermore, the SARS-CoV-2 receptor ACE2 was upregulated in these patients. This study conducted by Su et al. confirms once more the virus tropism for kidney tissue [40].

Menter et al. identified in patients who died with COVID-19 the presence of capillaritis and microthrombi in the lungs, and showed diffuse vascular damage in other organs highly suggestive of vascular dysfunction [41].

Cutaneous biopsies from the skin lesions associated with SARS-CoV-2 were also performed. The optical microscopy findings of a biopsy from a chilblain-like lesion in a 23-year-old patient diagnosed with coronavirus disease revealed the presence of inflammatory infiltrate, consisting especially of lymphocytes, which were "tightly cuffing the vessels" [42]. Kanitakis et al. accomplished histological,

immunofluorescence, and immunohistochemical studies in seventeen cases of acral chilblain-like skin lesions in patients with suspected, but not confirmed, coronavirus disease, and endotheliitis was present in 65% of cases [43]. The association of COVID-19 with chilblain-like skin lesions is still conflicting. Initially, acral lesions were thought to be related to SARS-CoV-2 infection, but more recent case studies could not sustain an association between them [43, 44].

All data collected from the autopsies indicate that changes in the endothelium are not limited to the lungs and suggest that COVID-19 is a whole-body disease.

Numerous symptoms of SARS-CoV-2-positive patients could be assigned to multiorgan endotheliitis and subsequent endothelial dysfunction.

As mentioned above, tropism for the kidneys, lungs, and cardiovascular system of the novel coronavirus was demonstrated. This explains the respiratory and cardiocirculatory events associated with the disease. Several hypotheses were proposed in order to explain other organ specific symptoms. The early neurological manifestations (hyposmia, anosmia, dysgeusia, or hypogeusia) which have been frequently described in these patients together with life threatening events such as stroke and intracerebral or subarachnoid hemorrhage could represent a consequence of endotheliitis [45]. In a short communication, Benger et al. made a detailed analysis of 5 patients with COVID-19 and intracerebral hemorrhage. They suggest that endothelial damage and endotheliitis along with a prothrombotic state and proinflammatory cytokine production are responsible for intracerebral hemorrhage, which occurred in younger individuals. Hemorrhage affected the anterior cerebral circulation [46].

In addition to the detrimental effect on blood vessels, the heart also represents a target for SARS-CoV-2. The main cardiovascular manifestations of COVID-19 are cardiac arrhythmias, caused by the inflammation of the myocardium and metabolic dysregulation [47]. It has been suggested that both direct and indirect viral injury is responsible for COVID-19-associated myocarditis [48].

The emerging evidence recognizes the endothelium as a key factor in the pathophysiological chain in COVID-19 [49]. Therefore, arterial and venous thrombosis, pulmonary embolism [49], central nervous system acute hemorrhagic events, and multiorgan failure associated with SARS-CoV-2 infection [50] might be the aftermath of subsequent endotheliitis and endothelial dysfunction associated with a procoagulant state. Endothelial cell damage together with endotheliitis also explains the predisposition for severe manifestations of the disease in patients with preexisting endothelial dysfunction caused by chronic pathologies such as hypertension [47].

While the major role of endothelial cells in the pathophysiology of COVID-19 is a compelling subject for ongoing research projects, the hypothesis according to which the endothelium could represent a therapeutic target in critically ill patients is intensely analyzed [49].

4. COVID-19-Renin-Angiotensin System

The role of the renin-angiotensin-aldosterone system (RAAS) in COVID-19 infection has been taken into consideration from the beginning of the pandemic, since one of the first known facts was that ACE2 (angiotensin-converting enzyme 2) is the receptor that allows SARS-CoV-2 to enter human cells.

RAAS is a natural protective mechanism for maintaining circulatory volume. Renal hypoperfusion stimulates renin release from the juxtaglomerular apparatus. Renin cleaves angiotensinogen to angiotensinogen I, and ACE hydrolyzes Ang I to Ang II. Ang II binds to angiotensin II type 1 receptor (AT1R) and promotes aldosterone production, leading to sodium retention, water reabsorption, and vasoconstriction. On the other arm of the cascade, ACE2 is maintaining the equilibrium by converting Ang II to angiotensin 1-7. Angiotensin 1-7 binds to the Mas receptor and mediates anti-inflammatory, antioxidative, and vasodilatory effects. In the case of insufficient ACE2, Ang II binding AT1R prevails and exerts vasoconstrictive and proinflammatory effects [51].

Angiotensin-converting enzyme 2 (ACE2) is expressed in the human vascular endothelium, respiratory epithelium, and other types of cells, and represents a primary mechanism for the entry and infection of SARS-CoV-2 virus. In a physiological state, ACE2 through the activity of carboxypeptidase generates angiotensin fragments (Ang 1-9 and Ang 1-7) and plays an essential role in the renin-angiotensin system (RAS), which is an important regulator of cardiovascular homeostasis. SARS-CoV-2 through its surface glycoprotein interacts with ACE2 and invades the host cells.

For SARS-CoV-2 infection, in addition to ACE2, one or more proteases including transmembrane protease serine 2 (TMPRSS2), basigin (also known as CD147), and potentially cathepsin B or cathepsin L are required [52].

ACE2 is expressed as a transmembrane protein whose active site is exposed at the extracellular surface and resides in the lung alveolar epithelial cells, heart, kidneys, vessels, and gastrointestinal system [53]. ACE2 can be cleaved and circulates in small amounts in the blood stream, but its role is uncertain [54–57].

While ACE2 is clearly responsible for facilitating cell insertion, it may also be the cause of individual variation in disease severity. The polymorphism of ACE2 in the population could impact the affinity for the virus's spike protein and make the infection more likely or more severe [57]. Also, the ACE2 gene is X-linked, and this could explain the slight protective effect in the female sex observed in COVID-19. Besides these genetic variations, ACE2 gene expression is increased in diabetes, CVD, and hypertension [58]. Several researches indicate that RAAS-modulating drugs could also modulate ACE2 expression and activity in various ways. Animal model studies have shown that ACE inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) upregulate ACE2 cell expression, and ARBs and mineralocorticoid receptor antagonists (MRA) increase ACE2 activity, [59, 60]. However, simultaneously, ACEIs reduce Ang II synthesis, and consequently, in the absence of excess Ang II, AT1R is thought to interact with ACE2 [61]. This interaction could reduce the affinity of COVID S protein to ACE2 and then reduce COVID-19 viral entry [61].

SARS-CoV-2 spike protein binding to ACE2 in alveolar epithelial cells downregulates ACE2 expression. Without ACE2 to lead Ang II to angiotensin 1-7, Ang II binds to AT1R, leading to a hyperaldosteronism state, materialized as hypokalemia in severe cases of COVID-19 infection [62], vasoconstriction, fibrosis, and inflammatory cell proliferation [63]. Murine studies proved that loss of ACE2 expression enhances vascular permeability, increases lung edema and neutrophil accumulation, and hence worsening lung function [64].

One of the earliest researches of Chinese scientists empowers the theory that excessive Ang II leads to a bad outcome. Liu et al. observed in a small cohort of COVID-19 patients that the plasma concentrations of Ang II were significantly higher than in healthy individuals and also that Ang II levels in COVID-19 patients were correlated with viral load and lung injury [65].

Besides exacerbated inflammation and hypoxemia through vasoconstriction in small pulmonary vessels, Ang II induces plasminogen activator inhibitor-1 (PAI-1) expression in endothelial cells via the AT1 receptor. PAI-1 leads to unresolved fibrin deposits in the alveoli of patients with both SARS and COVID-19 infection [51]. Also, excessive Ang II can be metabolized to angiotensin IV [66], which enhances thrombosis development [67, 68]; since hypercoagulability has been noticed in many severe cases, it can be hypothesized that a reduction in ACE2 contributes to increasing thrombotic risk.

Since ACE2 has been recognized as the gate of SARS-CoV-2, worldwide medical boards raised the question if RAAS modulators—ACEIs and ARBs—increase the risk of developing severe forms of COVID-19 infection. The rationale behind this concern was based on some experimental animal models which have shown increasing numbers of ACE2 after intravenous infusion of ACEIs and ARBs [59].

In order to establish whether RAAS modulators are harmful or not, scientists firstly compared the outcomes of COVID-19 patients with arterial hypertension and different treatments. Shyh et al. found that those on ARBs are significantly less likely to develop COVID-19, while ACEIs did not show a similar effect, considering that they do not directly affect ACE2 activity [69]. On the other hand, patients taking calcium channel-blockers (CCBs) had a significantly increased risk of manifesting symptoms of COVID-19.

Several other retrospective multicenter studies [63, 70] looked for an association between in-hospital use of ACEIs/ARBs and all-cause mortality of COVID-19 among patients with hypertension. Their results show that COVID-19 hypertensive patients treated with ACEIs/ARBs had a better outcome than COVID-19 patients without ACEIs/ARBs or treated with a different class of other antihypertensive agents. On a molecular basis, they identified that patients on ACEIs/ARBs had lower levels of IL-6, decreased cytokine production, and decreased viral load during hospitalization, and peripheral T cells were significantly higher than in the non-ACEI/ARB group [70].

Researchers' restless work not only offered substantial information about the role of ACE2 in COVID-19 infection, but also brought up several potential therapeutic approaches: spike protein-based vaccine, inhibition of transmembrane protease serine 2 (TMPRSS2-human proteinase which facilitates viral spike protein binding to ACE2) activity, blocking ACE2 receptor, and delivering an excessive soluble form of ACE2 [71]. It was postulated that delivering excessive soluble ACE2 would capture most of the viral load, restricting their fixation on cell membrane ACE2, and therefore limit the infection and also keep the balance of the 2 RAAS arms, preventing severe inflammatory tissue lesions [72, 73]. Most of these theories are based on animal model or in vitro studies and, needless to say, require extensive research and trials before becoming available therapies.

5. Cytokine Storm Associated with SARS-CoV-2 Infection

About 5% of the patients infected with SARS-CoV-2 develop critical disease forms manifesting by respiratory failure, shock, or multiple organ failure [74]. The presence of these disease forms does not seem to be correlated with viral load. Although these patients have a high viral load, the same load is found in patients having mild forms of the disease and even in asymptomatic persons [75]. Thus, the hypothesis was advanced that abnormal immune response, manifesting as a "cytokine storm," is the main determining factor of disease severity [76].

Cytokine storm associated with COVID-19 is similar to other clinical entities, such as cytokine release syndrome observed following CAR-T cell therapy [77], primary or secondary hemophagocytic lymphohistiocytosis (HLH), sepsis caused by Herpesviridae and other pathogens [78], and macrophage activation syndrome that occurs in various autoimmune diseases [79].

This progressive systemic inflammation leads to the loss of vascular tone clinically manifesting by a decrease in blood pressure, vasodilatory shock, and progressive organ failure. In the context of cytokine storms associated with highly pathogenic viruses such as SARS-CoV-2, SARS-CoV, and MERS-CoV, the greatest impact is on the lungs, where acute respiratory distress syndrome (ARDS) occurs which is the main cause of death. The effects are not limited to the lungs; cardiac, renal, and central nervous system damage is also involved [80].

After receptor binding and complex internalization, the viral RNA is released into the cell cytosol, replicated, and finally removed by exocytosis.

Intracellular viral RNA is identified by the recognition mechanisms of the innate immune response through specific receptors: PRRs (pattern recognition receptors), TLRs (toll-like receptors), and NLRs (NOD-like receptors). The recognition of viral RNA by these receptors determines the activation of intracellular signaling pathways, such as NF-κB and IRF 3/7. NF-κB stimulates the transcription of proinflammatory cytokines such as TNF-alpha, IL-6, and IL-1 and activates the immune response mediated by T helper 1 and 17 lymphocytes. IRF 3/7 stimulates the production of type 1 IFN, which induces activation of the JAK1/TYK2-STAT1/2 pathway, the effect being the transcription

of interferon-stimulated genes (ISG), with a role in the secretion of cytokines and the activation of other immune system components to stop viral replication [81, 82].

Previous studies have shown that in some cases, coronaviruses can delay type I IFN response through various mechanisms, the result being a more severe form of the disease caused by ineffective viral replication control and paradoxical hyperinflammation caused by type I IFN. In the case of SARS-CoV-2, an altered response of type I IFN seems to occur. A study showed that serum IFN activity was significantly lower in patients with severe or critical forms of the disease compared to those with mild-moderate forms. Moreover, serum ISG and type I IFN values in patients who subsequently developed ARDS with the need for invasive ventilation indicated that a mitigated type I IFN response precedes clinical deterioration [83].

This abnormal response of interferon leads to a massive inflow of neutrophils and monocytes, which are a major source of proinflammatory cytokines, apoptosis of T lymphocytes, and epithelial and endothelial cells [81].

Lymphopenia occurs in about 80% of the patients infected with SARS-CoV-2 and is more marked in the severe forms of the disease. There are many causal hypotheses explaining this process. Firstly, the virus can directly infect T lymphocytes but cannot replicate inside these, thus leading to cell death through apoptosis, necrosis, or pyroptosis. Secondly, the first wave of cytokines released, described above, includes anti-inflammatory cytokines such as TNF-alpha and IL-10, which cause apoptosis, exhaustion, and inhibition of TL proliferation. Not the least, lymphopenia could be the result of redistribution in the lungs and lymphoid organs [81, 84].

In the most severe disease cases, a sudden and rapid clinical deterioration occurs, which is associated with increased levels of acute phase reactants, coagulopathy, and cell lysis, and high proinflammatory cytokine levels, suggesting a second wave of cytokines, responsible for the so-called cytokine storm [81].

The triggering factor of the cytokine storm seems to be immunodeficiency caused by the decrease in the number and the dysfunction of T lymphocytes. Although other innate immunity hyperactivation mechanisms are supposed to be responsible, the cytokine storm is much more likely to occur as a result of a delayed response of innate immunity, followed by persistent hypercytokinemia and an abnormal response of the acquired immune system through T lymphocytes. The result is the failure to eliminate apoptotic cells or macrophages migrated to the site of inflammation and continuous antigenic stimulation by failure of viral clearance. These cells will continue to secrete proinflammatory cytokines, of which the most important are IL-18 and IFN-γ, which restimulate macrophage activation. Thus, a vicious circle is created which culminates in cytokine secretion, hemophagocytosis, coagulopathy, and ARDS [82, 85].

5.1. Cytokines and the Correlation with the Severity of the Disease. The first evidence of this correlation comes from the study conducted by Huang et al. in a sample of 41 patients who had the plasma levels of several cytokines and chemokines measured. The authors observed that the initial plasma levels of IL-1B, IL-1RA, IL-7, IL-8, IL-9, IL-10, FGF, GCSF, GMCSF, IFN-γ, IP-10, MCP1, MIP1A, MIP1B, PDGF, TNF-α, and VEGF were higher in all COVID-19 patients compared to healthy persons, the plasma concentrations of IL-5, IL-12p70, IL-15, eotaxin, and RANTES were similar in patients infected with SARS-CoV-2 and healthy persons, and the levels of IL-2, IL-7, IL-10, GCSF, IP-10, MCP1, MIP1A, and TNF-α were significantly higher in patients with severe forms of the disease requiring intensive therapy compared to those with mild or moderate forms [86]. Since then, many studies have been conducted in the attempt to elucidate the pathogenic mechanisms of the exacerbated immune response associated with SARS-CoV-2 infection and in the attempt to identify laboratory markers that correlate with the severity and prognosis of the disease in order to achieve a stratification of patients for adequate management based on early therapeutic intervention.

A recently published meta-analysis of 50 studies showed statistically significantly higher values of IL-2, IL-2R, IL-4, IL-6, IL-8, IL-10, TNF-α, and INF-γ in patients with severe forms of the disease compared to the others. In contrast, there were no significant differences between IL-17 and IL-1β values. As it can be seen, in some cases, there is an excessive production of proinflammatory as well as anti-inflammatory cytokines (IL-2R, IL-10), which highlights the dual pathogenic mechanism responsible for the occurrence of the cytokine storm [87]. Another meta-analysis and extensive systematic analysis shows that in patients with severe forms of the disease, lymphocytopenia (decreased CD3, CD4, and CD8 T lymphocytes), leukocytosis, high values of ESR, procalcitonin, LDH, and ALT occur more frequently. The levels of inflammatory cytokines, especially IL-6, 8, 10, and 2R and TNF-alpha, were significantly increased [88].

Regarding the profile of leukocytes, both meta-analyses evidenced a significant decrease in CD4 and CD8 T lymphocytes in the group of patients with severe disease forms [87, 88].

The most studied interleukin is perhaps IL-6, given that tocilizumab, a monoclonal antibody directed against the IL-6 receptor, can be used as therapy for COVID-19 patients who present signs of hyperinflammation. Mojtabavi et al. show in their analysis of 11 studies that IL-6 values are significantly higher in patients with severe forms of COVID-19 compared to those with mild or moderate forms [89]. Furthermore, Laguna-Goya et al. elaborated a model for predicting the risk of mortality in hospitalized COVID-19 patients based on IL-6 values. This includes 5 parameters: $FiO_2/SatO_2$ ratio, neutrophil/lymphocyte ratio, IL-6 value, LDH value, and age. This model might help to stratify patients into more uniform groups from a clinical and biological point of view before their inclusion in randomized clinical trials evaluating the efficacy of tocilizumab or other drugs. Until completion of clinical trials, this model could be used to select patients that would benefit the most from immunomodulatory therapy [90].

The prognostic value of IL-6 was also demonstrated in another study, where it was incorporated along with

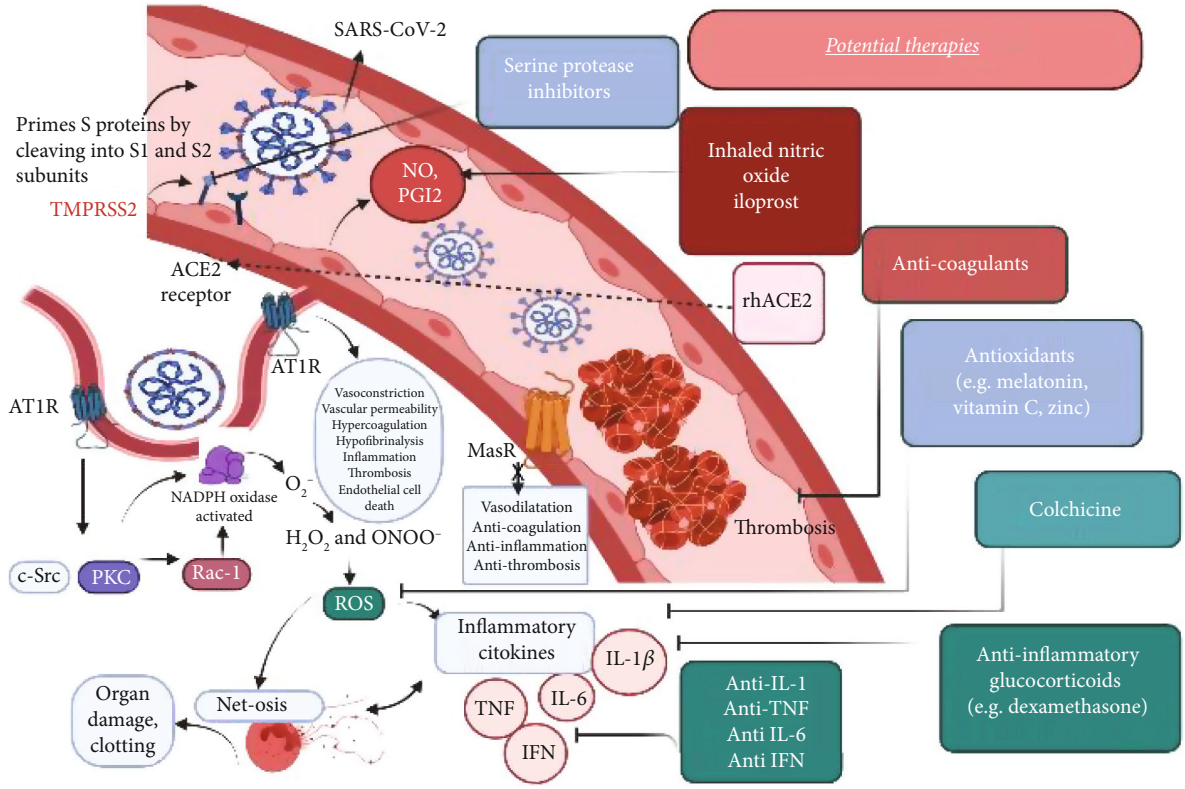

Figure 3: Mechanisms of endothelial dysfunction, inflammation, oxidative stress, and therapeutic targets in SARS-CoV-2 infection. SARS-CoV-2 infection begins when its peak proteins are proteolytically prepared by TMPRSS2, allowing them to bind to ACE2 and initiate viral endocytosis in the EC. This increases the amount of binding of Ang II to AT1R, which in turn activates NADPH-oxidase and subsequently induces an increased production of ROS. These excess ROS mediate signaling pathways that increase the production of inflammatory cytokines (such as IL-1β, IL-6, and TNF), decrease the bioavailability of NO and PGI2, and induce endothelial cell apoptosis, leading to endothelial damage and dysfunction. Furthermore, the release of proinflammatory and prothrombotic factors can lead to vascular inflammation, platelet aggregation, and thrombosis. These interactions increase the risk of thrombosis and lung damage in people infected with SARS-CoV-2. ROS also induce an overflow of NETs. There may be several positive feedback loops between cytokines (TNF-α, IL-1β) and ROS production as well as between cytokines (TNF-α, IL-1β) and NET formation. ROS, NETs, and proteolytic enzymes released by activated neutrophils also contribute to organ damage and clotting in vessels. Therapeutic targets address SARS-CoV-2-induced feedback loops in EC. Although there have been many therapies proposed to stop the spread of the coronavirus pandemic, those described here address feedback loops involving endothelial dysfunction, oxidative stress, and inflammation. TMPRSS2: transmembrane protease, serine 2; ACE2: angiotensin-converting enzyme 2; AT1R: angiotensin type 1 receptor; ROS: reactive oxygen species; c-Src: protooncogene tyrosine-protein kinase Src; PKC: protein kinase C; IL: interleukin; TNF: tissue necrosis factor; NO: nitric oxide; PGI2: prostaglandin I2 (also known as prostacyclin).

CD8+ TL into a prognostic model. The authors of the study showed that IL − 6 values > 20 pg/mL and CD8 + TL values < 165 cells/μL are correlated with mortality, being a better indicator of in-hospital mortality than the CURB-65 score [91].

Other cytokines were studied in the attempt to identify the prognostic factors of disease severity and prove their usefulness. An example is represented by IL-2R, included in several prognostic models such as the IL-2R/lymphocyte ratio, as demonstrated in the study conducted by Hou et al. [92], or the model developed by another group which incorporates IL-2R, the values of neutrophils, lymphocytes, and thrombocytes [93]. Another study proposes to monitor IP-10 and MCP-3 values early during the course of the disease in order to identify patients at risk for hyperinflammation and implicitly for more severe forms of the disease [94].

6. Therapeutic Targets for the Treatment of COVID-19

Numerous therapeutic targets (Figure 3) have been proposed taking into consideration the various mechanisms of action of SARS-CoV-2 on the endothelium. Regarding the key role of oxidative stress, endotheliopathy, and inflammatory mediators in the COVID-19 pathogenesis [8], we will further present the therapies that counteract the SARS-CoV-2-induced disturbances.

6.1. Interleukin-6 Inhibitors. As shown above, IL-6 plays an extremely important role in the occurrence and maintenance of the cytokine storm associated with COVID-19 and is correlated with disease severity, and thus it is an important therapeutic target. In addition, the inhibitors of IL-6 or its receptor proved to be effective in the treatment of other

similar syndromes such as HLH associated with Still's disease [95] or in the cytokine storm secondary to CAR-T cell therapy [96]. Regarding their use in COVID-19 patients, only data from case-control studies or case reports are currently available. It should be taken into consideration that these studies were extremely heterogeneous, performed on small samples, with divergent results concerning the monitored indicators (e.g., the need for invasive ventilation and the length of hospital stay). With respect to mortality, the majority showed an increase in survival or at least a favorable trend. Currently, many clinical trials are in progress to evaluate the efficacy and safety of using IL-6 inhibitors in this context. Experimental studies have shown that IL-6 can have a dual effect, both facilitating and suppressing viral replication [23], so that the optimal time of administration is another question that these clinical trials should answer [82, 97–100].

Tocilizumab, sarilumab, and siltuximab are Food and Drug Administration- (FDA-) approved IL-6 inhibitors evaluated for the management of patients with COVID-19 who have systemic inflammation. Tocilizumab is a recombinant humanized anti-IL-6 receptor monoclonal antibody that is approved by the FDA for use in patients with rheumatologic disorders and cytokine release syndrome (CRS) induced by chimeric antigen receptor T cell (CAR-T cell) therapy. Tocilizumab in combination with dexamethasone are indicated in certain hospitalized patients who are exhibiting rapid respiratory decompensation due to COVID-19 [101]. Further findings from REMAP-CAP and the RECOVERY study justify the use of tocilizumab in certain hospitalized patients with rapid respiratory decompensation due to COVID-19 [102].

Sarilumab is a recombinant humanized anti-IL-6 receptor monoclonal antibody that is approved by the FDA for use in patients with rheumatoid arthritis. It is available as an SQ formulation and is not approved for the treatment of CRS [101]. Preliminary efficacy results from REMAP-CAP for sarilumab were similar to those for tocilizumab. Compared to placebo, sarilumab reduced both mortality and time to ICU discharge, and increased the number of organ support-free days; however, the number of participants who received sarilumab in this trial was relatively small, limiting the conclusions and implications of these findings [102].

Siltuximab is a recombinant human-mouse chimeric monoclonal antibody that binds IL-6 and is approved by the FDA for use in patients with multicentric Castleman's disease. Siltuximab prevents the binding of IL-6 to both soluble and membrane-bound IL-6 receptors, inhibiting IL-6 signaling. Siltuximab is dosed as an IV infusion [103]. There are limited data describing the efficacy of siltuximab in patients with COVID-19 [104].

6.2. Interleukin-1 Inhibitors. Anakinra is a recombinant IL-1 receptor antagonist, currently approved in the treatment of a number of autoimmune diseases induced by excessive IL-1 secretion, with the aim of reducing inflammation and complications such as ARDS [105].

Starting from the data obtained from the use of anakinra in other similar syndromes such as secondary HLH or macrophage activation syndrome [105] and taking into consideration the high values of this interleukin reported in persons infected with SARS-CoV-2, it was supposed that IL-1 could be an important target in the management of the cytokine storm associated with SARS-CoV-2 as well. A retrospective study showed a clinical improvement in 72% of COVID-19 and ARDS patients treated with this drug [106]. Several randomized clinical trials that test anakinra in COVID-19 patients are underway.

Aside from anakinra, canakinumab, a high-affinity human monoclonal antibody [101], and rilonacept, a soluble IL-1 trap, represent therapeutic options for IL-1 inhibition [107].

Canakinumab counteracts the activity of IL-1 by blocking the interaction between IL-1β and its receptor [108]. The beneficial effect of canakinumab for COVID-19 patients results from the improvement of clinical status and reduction of invasive mechanical ventilation needed in these patients together with a prompt amelioration and maintenance in oxygenation levels [109, 110]. Furthermore, canakinumab ameliorates the prognosis of COVID-19 patients and prevents the clinical degradation by blocking the cytokine storm [110].

6.3. Anti-TNF-α. TNF-α is another cytokine with important inflammatory effects, whose increased serum values were also demonstrated in COVID-19 patients. Opinions diverge on the usefulness of anti-TNF-α monoclonal antibodies in this context. Infliximab, adalimumab, etanercept, certolizumab, and golimumab are the 5 most commonly prescribed TNFs inhibitors. On the one hand, TNF-α inhibition decreases IL-6 and IL-1 concentrations and reduces capillary permeability [111], and studies on animals have shown that the inhibition of this cytokine confers protection against SARS-CoV-2 infection. On the other hand, studies in which TNF-α inhibitors were used in syndromes similar to the cytokine storm have reported divergent results, some of them even demonstrating an aggravation of the disease [112].

6.4. Type I IFN. Considering the key role of IFN in antiviral response and its immunomodulatory effect, type I IFN seems to be an important potential therapeutic target. Type I IFN was studied both in vivo and in vitro, as monotherapy or in combination with antiviral drugs, in the treatment of SARS-CoV and MERS-CoV infection. Although interferon treatment was demonstrated to be efficient in vitro and in some studies on animals, in human studies the results were divergent. These results can be explained by the limited number of patients included and the heterogeneity of the studies, by the different inhibition mechanisms of the IFN signaling pathway used by the two viruses, as well as by the difficulty in assessing whether the clinical benefit observed was due to IFN or to the drugs with which it was used as part of combined therapy [113].

Another explanation for these results could be the subtype of IFN used as a therapeutic target. Compared to IFN-α, IFN-β seems to be a much more potent inhibitor of coronaviruses [114]. The time of administration seems to be an important element. Early administration was

associated with favorable results, while late administration was associated with significant adverse reactions without an effect on viral replication [115]. In addition, in vitro studies report viral replication inhibition by administration of prophylactic IFN in the case of SARS-CoV-2, while the same strategy is ineffective in the case of SARS-CoV and MERS-CoV [116–118]. A prospective study conducted in China on a sample of 2944 persons working in the health care system showed that interferon administered as a nasal spray is effective in the prophylaxis of SARS-CoV-2 infection [119].

Starting from the information obtained from previous studies on SARS-CoV and MERS-CoV and from the data regarding the pathology of SARS-CoV-2 infection, a number of clinical trials are in progress to test the efficacy of type I IFN in patients infected with SARS-CoV-2.

6.5. Inhibitor of Synthetic Serine Protease. Transmembrane protease serine 2 (TMPRSS2) represents the cornerstone in the SARS-CoV-2 S protein interaction with the endothelial cell [120]. TMPRSS2 is a protease that proved its capacity of preventing the cell invasion by SARS-CoV-2 in vitro [52].

Camostat mesylate, an inhibitor of synthetic serine protease infection, could block SARS-CoV-2 spreading in human tissue [120]. Taking into consideration the desirable effects in COVID-19 patients, TMPRSS2 has been approved for clinical use [52].

6.6. Recombinant Human ACE2 Protein (rhACE2). Taking into consideration that SARS-CoV-2 infection induces the depletion of ACE2 receptors, which contributes to systemic and especially pulmonary inflammation, the hypothesis was advanced that administration of recombinant human ACE2 protein can represent a therapeutic target. The causal mechanisms of immune dysfunction and hyperinflammation are multiple, so that the use of rhACE2 as monotherapy is probably insufficient, as demonstrated in patients infected with SARS-CoV in 2017 [76]. There is currently a clinical trial that studies the therapeutic efficacy of this molecule in COVID-19 patients.

6.7. JAK Inhibitors. The activated type I IFN JAK1/TYK2-STAT1/2 intracellular signaling pathway plays an important role in cytokine production, so that its inhibition might have a therapeutic effect in the cytokine storm associated with SARS-CoV-2.

Baricitinib is an inhibitor of JAK kinase currently used in the treatment of rheumatoid arthritis, which by selective and reversible binding to JAK receptors disrupts the transduction of the intracellular signal mediated by cytokines and thus attenuates the inflammatory response [121]. In addition, this compound is supposed to inhibit AAK1 receptor, required for viral endocytosis, also inhibiting in this way the entrance of the virus into the host cell [122].

At present, there are several ongoing clinical trials that investigate the efficacy of different JAK inhibitors in COVID-19 patients. An important aspect should be taken into account: the fact that SARS-CoV-2 infection predisposes to coagulopathy and formation of thrombi, and treatment with JAK inhibitors has been associated with an increase in thromboembolic risk [123].

6.8. Nitric Oxide. Inhaled nitric oxide (NO) proved its antiviral effects against various coronavirus strains together with the pulmonary vasodilation activity. Of great interest is the ability of NO in the prevention of the development of severe forms of the disease, if administrated at the proper time, at the early stage of COVID-19 [101].

6.9. Iloprost. The prostacyclin (PGI2) analogue, iloprost, showed beneficial effects in COVID-19 patients. Iloprost might represent a valuable therapeutic option for respiratory performance improvement [124]. Synthesized in the vascular endothelium, PGI2 plays a role not only in the endothelial barrier homeostasis and platelet aggregation, but it also has anti-inflammatory and vasodilatory effects. [125, 126].

In COVID-19 patients, iloprost could prevent the associated thrombotic events through its protective effects on the endothelium and the antithrombotic activity [124].

6.10. The Glycosaminoglycans. Another valuable therapeutic approach is represented by the glycosaminoglycans (GAGs), taking into consideration the double role they play in COVID-19 pathogenesis, their interaction with the chemokines, and the SARS-CoV-2 coreceptor function. Thus, the chemokine interaction with GAGs together with SARS-CoV-2 GAG-mediated cell entry might represent important targets in COVID-19 therapy [127].

6.11. Chemokine Receptor 5 Antagonism. The chemokine receptor 5 (CCR5) is a transmembrane structure expressed by several cells, including the endothelial cells [128], and it might be implicated in the SARS-CoV-2 invasion of the endothelial cells. By preventing the SARS-CoV-2 from entering the cell, the CCR5 antagonism could represent a valuable tool in preventing the severe inflammatory response characteristic for COVID-19-associated acute respiratory distress syndrome (ARDS) [127]. CCR5 antagonists proved their efficiency for preventing HIV-1 entry into the cells [129]. Maraviroc, a CCR5 antagonist, blocks the SARS-CoV-2 fusion with other cells (via S protein) and prevents its multiplication [130]. Leronlimab is a monoclonal IgG4 antibody which also has CCR5 as a therapeutic target. Leronlimab successfully reduced the IL-6 levels in patients with severe COVID 19 manifestations [131]. Taking into consideration the role of CCR5 in the COVID-19 pathogenesis and their expression by the endothelial cells, the CCR5 antagonism might represent a therapeutic option in the treatment of SARS-CoV-2-induced endotheliopathy.

6.12. The CXCL-8 Pathway. CXCL-8/IL-8 is an inflammatory chemokine that promotes the angiogenesis on endothelial cells via VEGF [132, 133]. The implication of the CXCL-8 pathway in SARS-CoV-2 infection pathogenesis results from its increased circulating levels identified in COVID-19 patients [134]. CXCL-8 is a powerful neutrophil chemotactic factor [135] and its high serum levels in COVID-19 patients might explain the associated neutrophilia. The neutralizing IL-8 antibody therapy and CXCL-8 receptor

(CXCR-2) antagonists might represent a therapeutic option for hospitalized COVID-19 patients [127].

7. Conclusions

This review summarized the relationship between COVID-19, endothelial dysfunction, inflammation, and oxidative stress. The implication of endothelium in SARS-CoV-2 pathogenesis remains a subject of interest which is intensely researched in current studies. Even though several studies place the endothelial dysfunction and oxidative stress as the main factors responsible for microvascular COVID-19-associated complications, the direct invasion of endothelial cells by SARS-CoV-2 remains disputable. An explanation for the severe COVID-19 manifestations in patients suffering from cardiovascular and metabolic comorbidities might be the endothelial dysfunction associated with the aforementioned conditions; thus, those patients are at high risk for developing pulmonary and extrapulmonary complications. The central role of endothelium in the COVID-19 pathogenesis remains of great interest particularly for its role as a valuable therapeutic target for the prevention and/or treatment of vascular complications in SARS-CoV-2 patients. With a plethora of physiopathological mechanisms, the SARS-CoV-2-induced endotheliopathy appears to play a central role in COVID-19 pathogenesis.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

The authors Adriana Fodor, Brandusa Tiperciuc, and Cezar Login have equal contribution.

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