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# Angiogenesis in Adipose Tissue: How can Moderate Caloric Restriction Affects Obesity-Related Endothelial Dysfunction?

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## Abstract

The plasticity of adipose tissue (AT) is related to its angiogenic ability. Angiogenesis is a multistep process which involves endothelial cell (EC) proliferation, migration, invasion and finally tube formation. AT as a secretory organ produces adipokines, which contributes to the development of subclinical inflammation. The inflammation-related adipokines deteriorate EC function and in consequence change the production of endothelial mediators responsible for vascular homeostasis and angiogenesis, leading to cardiovascular diseases (CVD) in obese patients. Additionally, the recent observation suggests that AT is poorly oxygenated in obesity. Hypoxia limits the healthy expansion of AT and stimulates a molecular response, enhancing nuclear factor kappa-B (NF- $\kappa$ B) and hypoxia-inducible factor (HIF-1) expression. HIF-1 $\alpha$  induction does not start a normal angiogenic process but rather induces inflammatory response and fibrosis that is strongly associated with insulin resistance (IR). It is believed that EC dysfunction in obesity can be reduced by caloric restriction (CR). Moderate CR reflects a real-life situation and could be optimal to achieve an EC improvement. It reduces adiposity leading to pro-angiogenic, anti-inflammatory and—to a lesser extent—anti-oxidative cellular effects, which not only preserves the healthy EC phenotype but also leads to an improvement of AT remodeling and prevent systemic IR.

**Keywords:** obesity, angiogenesis, endothelial dysfunction, adipokines, caloric restriction

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## 1. Introduction

Obesity (adiposity) is a serious health problem, especially in well-developed countries. The regional distribution of fat determines our health. Excessive accumulation of fat in the upper body's region (central obesity) is a stronger predictor of morbidity than excess fat in the lower

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body [1]. Central obesity is associated with insulin resistance (IR) and this condition predisposes to cardiovascular disease (CVD) [2]. Adipose tissue (AT) is not only an energy storage organ but also produces adipokines, which contribute to the development of subclinical inflammation [3]. The compounds released from AT are capable of affecting endothelial cell (EC) functions [4]. The mechanism of obesity-induced endothelial dysfunction is multifactorial mainly due to the omnidirectional impact of various adipokines, leading to the following abnormalities such as elevated blood pressure, formation of atherosclerotic plaques, oxidative stress, prothrombotic state and alterations in glucose and lipid metabolism [5]. AT remodeling is pathologically accelerated in an obese state due to local hypoxia. Reduced angiogenesis is a severe immune cell infiltration with subsequent pro-inflammatory responses which additionally deteriorates EC functions [6]. Therefore, one of the main goals of therapeutic interventions in obesity is to correct abnormalities in EC function and to protect endothelial integrity.

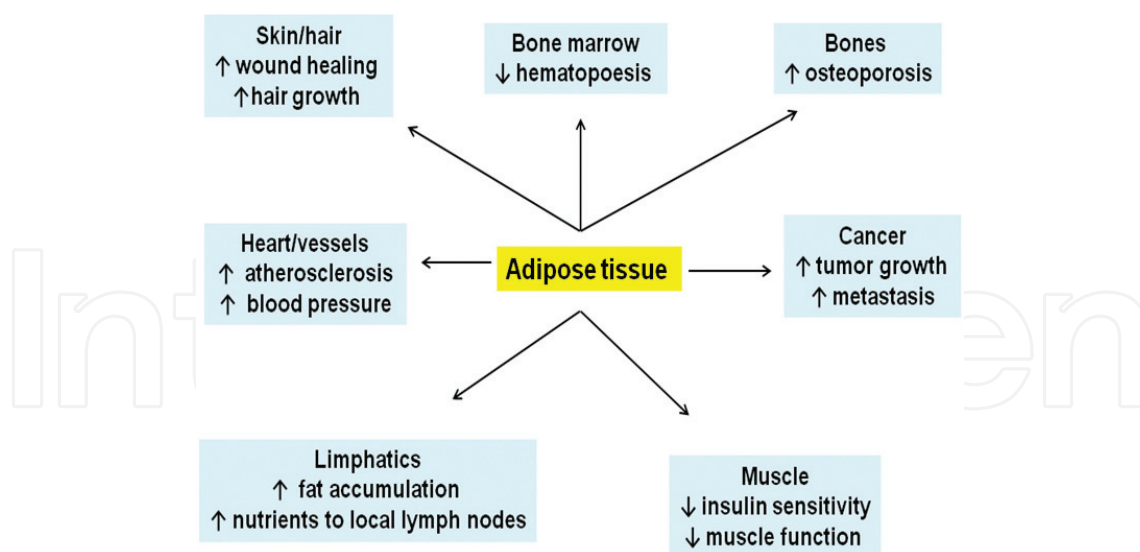
It is believed that EC dysfunction in obesity can be reduced by caloric restriction (CR), but it is unclear whether this benefit requires significant or moderate weight loss. In recent studies conducted on overweight humans, short- and long-lasting CR (6–52 weeks) have shown to improve a number of health outcomes [7–9]. The important issue is that most individuals have difficulty sustaining prolonged CR and the improvement of EC function may be problematic to achieve. Our cooperation with physicians, dieticians and psychologists allows us to claim that it is usually optimal for obese patients if CR is not so burdensome and yet, at the same time, effective. Therefore, we propose a mild CR as a way to lose body weight in obese individuals. Such a type of CR reflects a real-life situation and seems to be optimal to achieve an improvement of EC.

## 2. Adipose tissue

AT was earlier characterized as a connective tissue which stores triglycerides. An increase in global obesity and diabetes has attracted great interest to the function of this tissue. Nowadays, AT is considered an important regulator of energy balance, which plays a major role in nutrient homeostasis after feeding and releases free fatty acids (FFAs) during fasting. As such, it is regarded as an endocrine organ producing adipokines, which affect many organs and thus the homeostasis in the body (**Figure 1**).

AT is mainly found in subcutaneous and visceral depots. In obesity, AT is accumulated in different organs, including heart, liver, kidneys, bone marrow, lungs and the adventitia of major blood vessels, where secreted adipokines affect their function. Excess visceral adiposity is strongly correlated with IR, hypertension and dyslipidemia, which contribute to high rates of mortality and morbidity [3, 10]. Most adipokines, which stimulate inflammatory responses, are dysregulated in obesity and promote obesity-induced metabolic dysfunction, leading to CVD. The production of pro-inflammatory cytokines by AT is upregulated in an obese state (**Table 1**) while the secretion of anti-inflammatory factors is reduced (**Table 2**).

Adipocytes are divided into two types: white adipocytes and brown adipocytes. Brown adipocyte tissue (BAT) converts nutrients into chemical energy in the form of heat. Brown fat cells



**Figure 1.** The impact of adipose tissue on multiple organs mediated by various factors released from adipocytes [3].

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**Pro-inflammatory adipokines**

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Adipokine	Function
Leptin	Appetite control through the central nervous system
Resistin	Induces insulin resistance by stimulating the Il-6 and TNF- $\alpha$ production in macrophages
Retinol-binding protein 4 (RBP4)	Induces insulin resistance by influencing glucose homeostasis
Lipocalin 2 (neutrophil gelatinase-associated lipocalin)	Promotes insulin resistance through TNF- $\alpha$ secretion from adipocytes
Angiopoietin-like protein 2 (ANGPTL-2)	Activates inflammatory response in endothelium and promotes insulin resistance
Tumor necrosis factor- $\alpha$ (TNF- $\alpha$ )	Attenuates insulin signaling in muscles and adipose tissue (IR)
Interleukin 6 (Il-6)	Involved in insulin resistance. It has various function in different organs
Interleukin 18 (IL-18)	Inflammation, involved in plaque instability and endothelial activation
Monocyte chemoattractant protein 1 (MCP-1/CCL-2)	Inflammation, involved in monocyte chemotaxis
CXC-chemokine ligand 5 (CXCL5)	Secreted from macrophages in AT. Responsible for IR
Chemerin	Inflammation, involved in monocyte chemotaxis and stimulates lipolysis
Nicotinamide phosphor ribosyltransferase (NAmPT, Visfatin)	Modulator of B cell differentiation, correlates with visceral adiposity, monocyte chemoattractant

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**Table 1.** Pro-inflammatory adipokines and their functions [11, 12].

express a unique thermogenic and mitochondrial genetic program that promotes mitochondrial biogenesis, energy uncoupling and energy dissipation, in turn providing essential heat to the organism. Energy dissipation is possible in the presence of large amounts of mitochondria

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**Anti-inflammatory adipokines**


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<b>Adipokine</b>	<b>Main function</b>
Adiponectin	Negatively correlates with inflammation and visceral fat accumulation, protects against metabolic dysfunction and IR
Secreted frizzled-related protein 5 (SFRP 5)	Anti-inflammatory, important for insulin sensitivity
Visceral adipose tissue-derived serine protease inhibitor (Vaspin)	Positively correlates with BMI and insulin sensitivity, increases glucose tolerance
Omentin-1	Expressed in visceral fat, protects against IR. Its level is reduced in obesity
Apelin	It plays different functions in various organs. Its production is enhanced by insulin. Has angiogenic and hypotensive properties

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**Table 2.** Anti-inflammatory adipokines and their functions [11, 12].

with the uncoupling protein-1 (UCP-1). This provides heat rather than adenosine triphosphate (ATP) production [13].

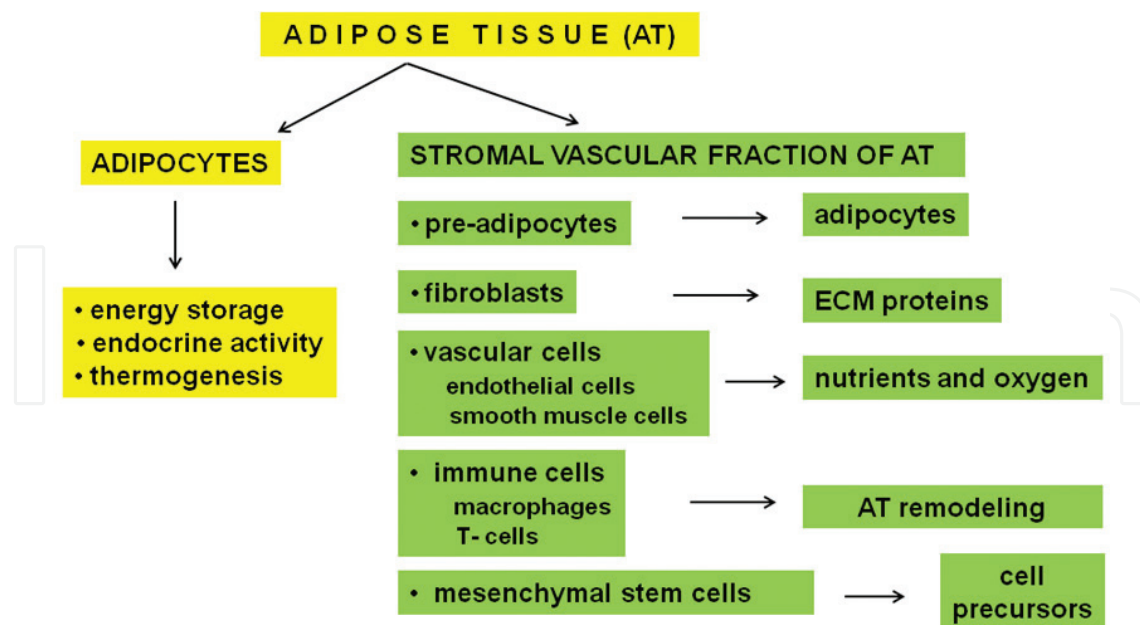
White adipose tissue (WAT) stores triglycerides during energy consumption and releases fatty acids during starvation. WAT is also an active endocrine organ that secretes a large number of adipokines. Adipokines act centrally to regulate appetite and energy expenditure. They peripherally affect insulin sensitivity, promote subclinical inflammation and lipid uptake and accommodate the conversion of steroid hormones. Fats can be classified as subcutaneous or visceral. WAT has a specific morphology. Histologically, subcutaneous fat contains mature large adipocytes, whereas visceral fat consists of small adipocytes. Subcutaneous and visceral depots contribute to metabolism in different ways. An increased subcutaneous fat deposition in the form of “pear-shaped” or female pattern of distribution might protect against certain aspects of metabolic dysfunction, especially against IR [14, 15]. However, visceral depots, in an “apple” or male pattern of distribution, are thought to be associated with metabolic complications and appear to increase the risk of diabetes, hyperlipidemia and CVD [16]. It has become popular to term subcutaneous adipose as ‘good fat’ and visceral as ‘bad fat’.

### 3. AT remodeling in obesity

#### 3.1. Infiltration of immune cells into AT

AT is mainly comprised of adipocytes, although other cell types contribute to its growth and function. These include pre-adipocytes, lymphocytes, macrophages, fibroblasts and vascular cells (**Figure 2**). AT can respond rapidly and dynamically to nutrient deprivation and also to its excess. One of the unique attributes of AT is its incredible capacity to change its dimensions. This effect can be accomplished by increasing the size of adipose cells (hypertrophy) or by recruiting new adipocytes from the resident pool of progenitors (hyperplasia).

AT expands first by hypertrophy until a critical threshold is reached, upon which signals are released for the induction of preadipocyte proliferation and differentiation (hyperplasia) [17].



**Figure 2.** Components of adipose tissue [3, 12]. *Abbreviations:* AT, adipose tissue; ECM, extracellular matrix.

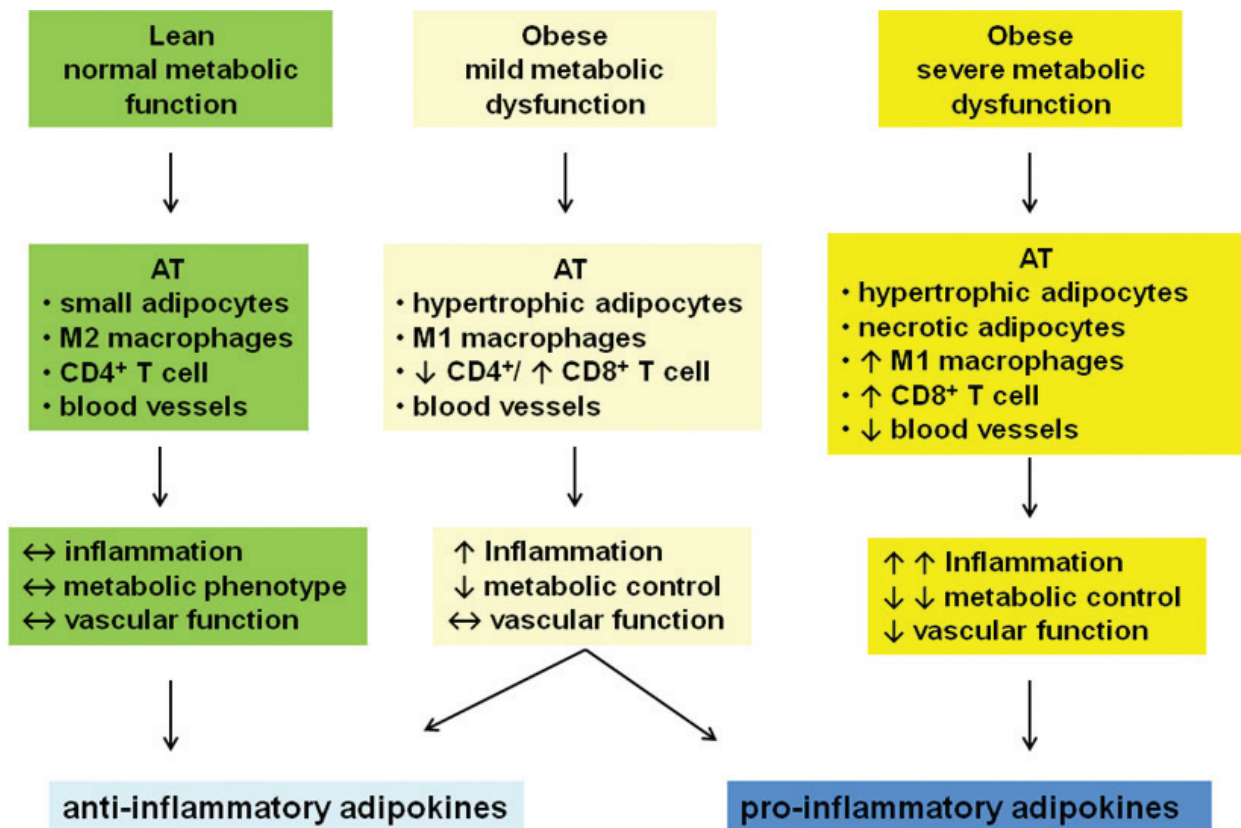
AT remodeling is pathologically accelerated in an obese state with reduced angiogenesis, extracellular matrix (ECM) overproduction and severe immune cell infiltration with subsequent pro-inflammatory responses. The large infiltration of macrophages in AT is linked to a systemic inflammation and IR. Moreover, the accumulation of macrophages is proportional to adiposity, and a sustained weight loss results in the lowering of inflammation, which suggests that this infiltration is reversible. Macrophages are also more abundant in the visceral than subcutaneous AT [6, 12]. Resident adipose macrophages display remarkable heterogeneity in their activities and functions. Hypertrophic adipocytes produce chemotactic factors, which promote monocyte accumulation in AT.

Macrophages can be classified into two broad groups: M1 and M2, based on the expression of particular antigens. Lumeng et al. proposed a model which emphasized that obesity is accompanied by a transformation of M2 anti-inflammatory macrophages (that are primarily accumulated during a negative energy balance) to more pro-inflammatory M1 macrophages [18]. The subsets of T cells presented in AT have been seen to be implicated in the macrophage activation. T helper cells (CD4<sup>+</sup>) are present in a large numbers in the AT of lean persons and have a protective effect by impeding M1 macrophages, resulting in increased insulin sensitivity. T cytotoxic cell (CD8<sup>+</sup>) can start the mobilization and activation of M1 macrophages and in this way it promotes an inflammation associated with IR. The M1 population positively correlates with IR and is characterized by overnutrition, where FFAs stimulate its pro-inflammatory responses [18]. In a lean state, resident macrophages are polarized toward the M2 state, which expresses a combination of anti-inflammatory factors that may help to preserve the normal adipocyte function by promoting AT repair and angiogenesis. Conversely, M1 macrophages induced by pro-inflammatory mediators express a repertoire of pro-inflammatory factors, which include tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 6 (IL-6), inducible nitric oxidase synthase (iNOS) and produce reactive oxygen species (ROS) [3, 6]. The

key function of macrophages is to remove apoptotic cells in an immunologically silent manner to prevent the release of harmful substances. The presence of apoptotic adipocytes surrounded by M1 macrophages (forming the so-called crown-like structures) is a characteristic feature in the obese with a full metabolic dysfunction. This pro-inflammatory state in AT is due to an impairment of the macrophage-mediated phagocytic process. The fibroblasts from metabolically dysfunctional AT produce excess ECM components that may contribute to metabolic dysregulation. The intercellular communication within AT is required for normal metabolic function. The obesity-associated changes in the cellular composition of AT lead to a modification of adipokine secretion [18, 19]. Consequently, obese patients can be categorized into those that have a fully dysfunctional metabolic phenotype and those that have a mildly dysfunctional metabolic phenotype (**Figure 3**) [19].

### 3.2. Angiogenesis in AT

Angiogenesis plays a central role in various physiological processes in a human body, not only during fetal development. Angiogenesis can be a hallmark of wound healing, menstrual cycle, cancer and various ischemic and inflammatory diseases. The pivotal process of angiogenesis can be simply described in multiple steps. First, angiogenic stimuli cause an increase in EC permeability and proliferation. Second, the proteolysis of the basement membrane components is a necessary process to promote the invasion of EC into the stroma of the neighboring



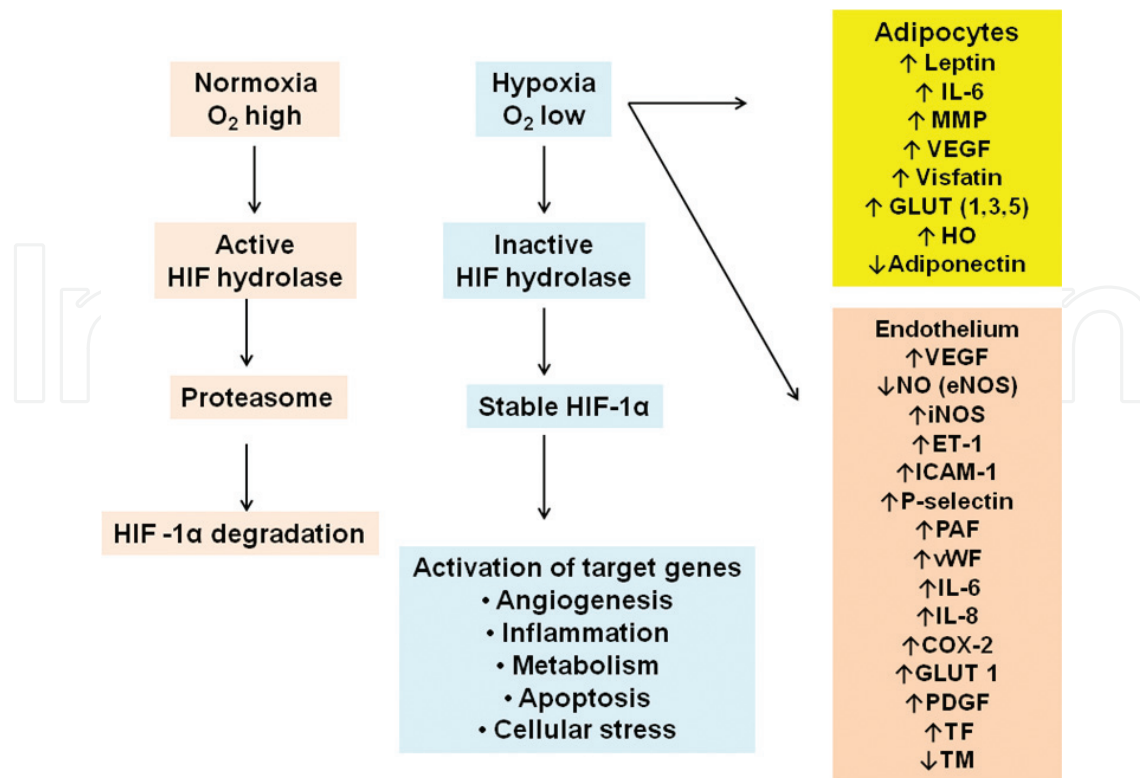
**Figure 3.** Adiposity-related metabolic dysfunction [3, 12]. *Abbreviations:* AT, adipose tissue; CD4<sup>+</sup>, T helper cell; CD8<sup>+</sup>, T cytotoxic cell; ↔, without changes.

tissue, in which the supportive activity of the tissue plasminogen activator system (t-PA and uPA—urokinase-type plasminogen activator) and matrix metalloproteinases (MMPs) are required. Third, the migrated ECs trigger lumen formation as the sprout forms a multicellular structure. Finally, the capillary is stabilized through the construction of a basement membrane, an adherent junction and ECs [20, 21].

AT possesses a relatively dense network of blood capillaries, ensuring an adequate exposure to nutrients and oxygen. The AT vasculature serves to transport systemic lipids to their storage depot in adipocytes, and transfers factors (e.g. adipokines) and nutrients (e.g. FFAs) from these cells in times of metabolic need. The microvasculature of AT is necessary for the expansion of adipose mass not only to prevent hypoxia, but also as a potential source of adipocyte progenitors in WAT. The blood capillary network also contributes to immunity and inflammation. AT macrophages serve multiple functions: (i) removal of necrotic adipocytes, (ii) production of pro-inflammatory and (iii) pro-angiogenic mediators [3, 22]. Obesity reduces the density of capillaries in AT, leading to localized hypoxia. The effect of hypoxia in obesity is complex and could be explained by: (i) the proportion of the cardiac output and blood flow that goes to WAT is not increased in the obese despite the expansion of the tissue mass, (ii) obese subjects do not exhibit the postprandial increase in the blood flow to AT that occurs in lean subjects and (iii) hypertrophied adipocytes are larger than normal, which impedes oxygen delivery to fat cells. Tissue hypoxia drives many cellular and molecular mechanisms. The first cellular mechanism responsible for local inflammation is macrophages recruitment. The necrosis of adipocytes, driven by hypoxia, is a prominent phagocytic stimulus that regulates macrophages infiltration. The second mechanism responsible for adipose inflammation is lipotoxicity. FFAs released from hypertrophic adipocytes could be transported to the liver and stored in lipid droplets. They could also be re-esterified to triglycerides in adipocytes. Those which escape re-esterification play a critical role as a primary energy source in several organs during prolonged fasting. FFAs are also ligands for TLR 4 (Toll-like receptor) presented in macrophages. FFAs binding with TLR 4 activate the inflammatory signaling cascade (NF- $\kappa$ B—nuclear factor kappa-B). The third mechanism is directly associated with oxygen deprivation (**Figure 4**) [22–24].

Hypoxia in AT has been investigated in human and animal models. Many adipokines related to inflammation (leptin, TNF- $\alpha$  and Il-6), MMPs, growth factors (VEGF—vascular growth factor and bFGF—basic fibroblast growth factor) are elevated in hypoxia [26]. The master regulator of hypoxia is hypoxia-inducible factor (HIF-1). It is a heterodimer composed of an oxygen-sensitive HIF-1 $\alpha$  subunit and a constitutively expressed HIF-1 $\beta$ , which is not directly regulated by oxygen. A substantial number of genes are recognized to be hypoxia sensitive. The target genes include those involved in angiogenesis, cell proliferation, survival, apoptosis, vascular tone, glucose and energy metabolism. The genes, which regulate leptin, VEGF and MMPs expression, are controlled by HIF-1 and become elevated in response to low oxygen partial pressure (pO<sub>2</sub>) in adipocytes. At the same time, the adiponectin gene is downregulated [27]. Glucose uptake by human adipocytes is strongly stimulated by hypoxia, presumably as a consequence of an increased amount of glucose transporters (GLUT). This may result in changes in insulin sensitivity. An experimental model of intermittent hypoxia has been shown to induce IR [28]. The effect of hypoxia on the WAT function has been discussed in terms of adipocytes, reflecting the fact that these are the cells that are characteristic of AT. Adipocytes





**Figure 4.** The response of adipocytes and endothelium to hypoxia [25, 26]. *Abbreviations:* HIF, hypoxia-inducible factor; IL-6, interleukin 6; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; GLUT, glucose transporter; HO, heme oxygenase; NO, nitric oxide; iNOS, inducible nitric oxide synthase; ET-1, endothelin 1; ICAM-1, intercellular cell adhesion molecule-1, PAF, platelet-activating factor; vWF, von Willebrand factor; IL-8, interleukin 8; COX-2, cyclooxygenase 2; PDGF, platelet-derived growth factor; TF, tissue factor; TM, thrombomodulin.

generally account for no more than 50% of the total cell content of WAT (**Figure 2**). The other non-adipocyte cells such as immune cells, vascular cells and pre-adipocytes are also affected by hypoxia, producing inflammatory mediators. There are several transcription factors which are implicated in molecular response to hypoxia, including NF- $\kappa$ B, which modulates the transcription of target pro-inflammatory genes. However, the pivotal role in response to hypoxia is played by HIF-1, which leads to proper angiogenesis. Hypoxia promotes angiogenesis by stimulating VEGF production in ECs, which plays a central role in angiogenesis and neovascularization. It is a potent mitogen for vascular ECs. It also releases other mitogenic molecules (PDGF—platelet-derived growth factor, bFGF, ET-1—endothelin-1) for smooth muscle cells and many pro-inflammatory mediators (IL-6, IL-1 $\alpha$ —interleukin 1 $\alpha$ , IL-8—interleukin 8, MCP-1—monocyte chemoattractant protein, iNOS) modulating the angiogenesis process [29].

Hypoxia can also act adversely by inhibiting the angiogenic response and by promoting EC death and apoptosis [30, 31]. The two major responses of ECs have been observed depending on the degree and duration of oxygen deficiency. Firstly, acute hypoxia rapidly activates the ECs to release chemoattractants (IL-8, PAF—platelet activating factor and MCP-1). This is a direct process which does not need gene induction. These inflammatory mediators are able to recruit and promote the adherence of leukocyte and platelets to endothelium, leading to a local

inflammatory reaction in ischemic tissue. Secondly, longer periods of hypoxia increase the expression of specific genes encoding cytokines, growth factors and pro-coagulation molecules by HIF-1 activation [25]. Hypoxia in EC also induces NF- $\kappa$ B activation. This promotes the synthesis of pro-inflammatory cytokines, prostaglandins and adhesion molecules, which supports the further transmigration of leucocytes to AT. The adverse effect of NF- $\kappa$ B expression in hypoxia is EC death and apoptosis (**Figure 4**) [31].

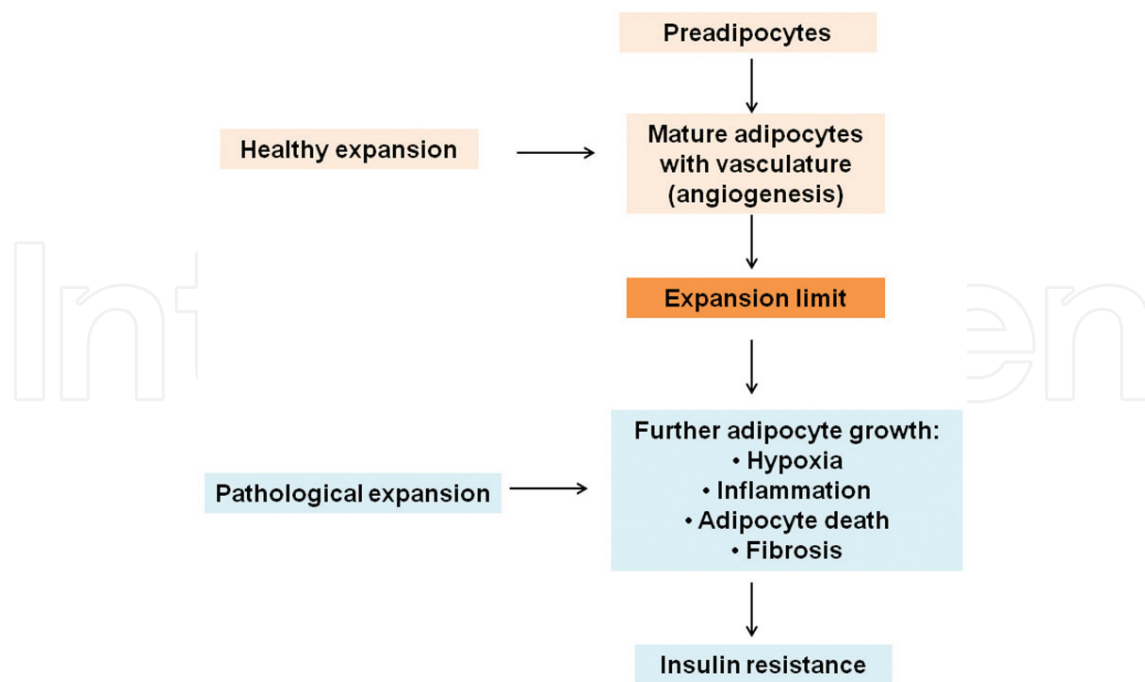
EC proliferation and migration, crucial for angiogenesis, could also be affected by hypoxia. The expression of VEGF and its receptor Flt-1 are upregulated by hypoxic endothelium. Both VEGF and its receptor Flt-1 are responsible for the strong mitogenic response in a hypoxic condition. In spite of VEGF overexpression, hypoxia can also paradoxically inhibit the angiogenic response, which could be blocked by a soluble form of VEGFR1 (Flt-1) [29, 30].

Hypoxia can also affect vascular tone, favorable for vasoconstriction. The basal and stimulated nitric oxide (NO) release by endothelium is quickly inhibited by hypoxia. This seems to be due to a decrease in the constitutive endothelial NO synthase (eNOS) expression and the concomitant increase in ET-1 release. In conclusion, the increased production of different mitogens combined with the suppression of endothelial NO would be expected during vascular remodeling [32]. Additionally, hypoxia increases the procoagulant activity, which correlates with a marked decrease of thrombomodulin (TM) and an increase in the tissue factor (TF) expression (**Figure 4**) [25].

Angiogenesis plays a critical role in healthy AT expansion. To better understand this issue, the overexpressed HIF-1 $\alpha$  in adipocytes in a transgenic mouse model was analyzed during hypoxia [33]. It was observed that there was no expression of the classical HIF-1 $\alpha$  target genes such as VEGF, or any components of angiogenic or anaerobic glucose pathway was registered. Surprisingly, scientists observed fibrosis, which was induced by the upregulation of lysyl oxidase (LOX), elastin, collagens (I, III) and the tissue inhibitor of MMP-1 (TIMP-1). They proposed a hypothesis that the accumulation of ECM in WAT during hypoxia causes local fibrosis with a subsequent inflammatory response and IR [33]. Briefly, a healthy AT expansion consists of adequate angiogenic response and appropriate remodeling of the ECM. In contrast, a pathological AT expansion consists of a massive enlargement of existing adipocytes, reduced angiogenesis and consequent hypoxia [33, 34] (**Figure 5**). It has been reported that obese mice receiving anti-angiogenic reagents have a reduced body weight while their adipose mass shows increased metabolic rates [35]. This is due to the fact that there is a close interplay between adipogenesis and angiogenesis in obesity [36].

In the end of this section, it is worth to mention about some important angiogenic and angiostatic factors crucial for appropriate angiogenesis. Obesity is known to modify these mediators [37]. Below it is shortly discussed the essence of action of pro-angiogenic factors such as bFGF, IGF-1 (insulin growth factor-1) and Ang-1 (angiopoietin-1) and angiostatic factors such as TSP-1 (thrombospondin), endostatin, Ang-2 (angiopoietin-2), IP-10 (interferon-induced protein) and IFN- $\gamma$  (interferon- $\gamma$ ).

bFGF is another essential pro-angiogenic factor besides VEGF. It changes ECs morphology, increases proliferation, migration and production of metalloproteinases which facilitates the



**Figure 5.** Healthy and unhealthy adipose tissue expansion [6, 26].

degradation of ECM. The autocrine secretion of bFGF by ECs is crucial for their migration and invasiveness [38]. Tsuboi et al. found correlations between bFGF and metalloproteinases in endothelial culture medium and suggested that expression of metalloproteinases is critical for migration and invasiveness of ECs and finally in the tube formations [39]. The clinical data analyzing the correlation between bFGF and abdominal obesity are still inconclusive [40, 41].

IGF-1 also called somatomedin C, has similar structure to insulin and possesses the affinity to insulin receptor. It is produced in the liver in response to growth hormone stimulation. As a mitogenic and anabolic factor, its effect is particularly important for the muscle, neural, hepatic, renal, lung and hematopoietic cells [42]. Additionally, the reduction of IGF-1 in rodents but not in humans is one of the most important effects of CR, which explains the maintenance of animal lifespan [43].

The key angiogenesis processes such as proliferation and migration are regulated by anti-angiogenic TSP-1. Bagavandoss and Wilks documented the anti-angiogenic effects of TSP-1 in various types of ECs, emphasize that its anti-angiogenic effect is mainly due to the inhibitory effect of endothelial proliferation [44]. Nowadays, TSP-1 is also classified as adipokine secreted by visceral fat, predisposing to IR and subclinical inflammation [45].

Endostatin is an endogenous inhibitor of angiogenesis, altering the action of VEGF and bFGF. The N-terminal sequence of this inhibitor is identical with a C-terminal fragment of XVIII collagen, presented in the basal membrane and extracellular matrix. Endostatin inhibits the proliferation, migration, adhesion and ability to tube formation. It blocks multiple signaling pathways, such TNF- $\alpha$  and NF- $\kappa$ B pathways, adhesion and also clotting process [46, 47]. Endostatin administration may reduce adipose tissue growth in animal model [35].

Maturation and stabilization of the blood vessels in the final stages of angiogenesis are controlled by a pair of opposing proteins—Ang-1 and Ang-2 [42]. Both proteins bind to the same Tie-2 (angiopoietin tyrosin kinase receptor) receptor on the surface of ECs resulting in opposite effects: Ang-1 acts as agonist and Ang-2 acts as antagonist. Ang-1 is secreted by adipocytes and Ang-2 by ECs [48]. Ang-1 concentration correlates with the percentage of adipose tissue in the body [49].

IP-10 is a chemotactic factor for T lymphocytes, produced by various cells such as monocytes, endothelium and fibroblasts in response to IFN- $\gamma$  stimulation [50]. IP-10 overexpression occurs in subcutaneous fat tissue in obese patients [51], but no differences between obese patients with or without diabetes were reported [52].

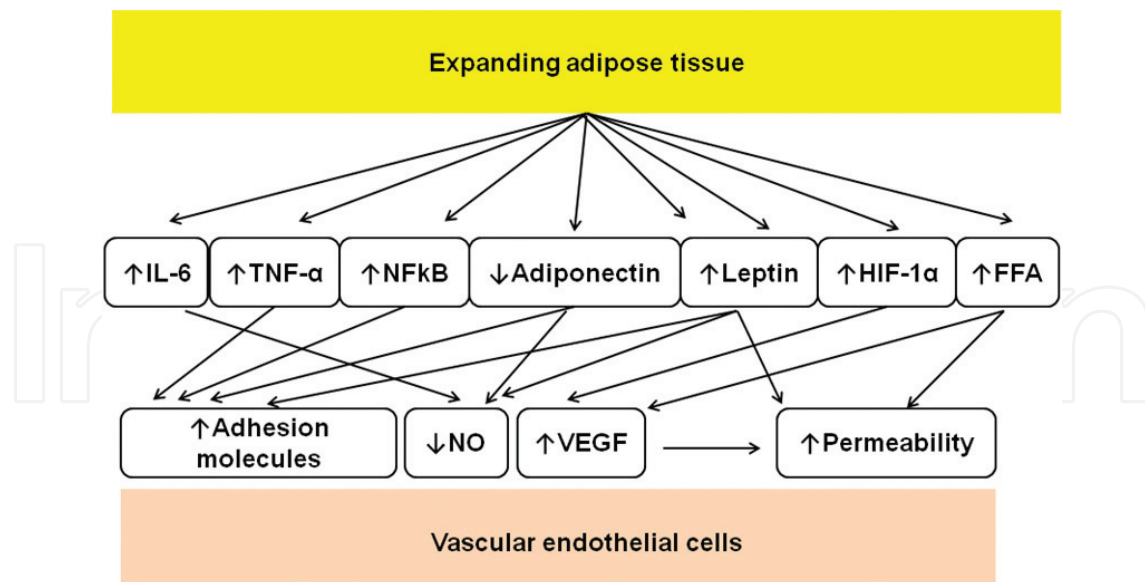
Infiltrating macrophages and lymphocytes are an important cause of inflammation and IR in AT [3, 18, 19]. IFN- $\gamma$  produced by lymphocytes changes the phenotype of macrophages to more pro-inflammatory—M1 [53]. Central obesity especially predisposes to high IFN- $\gamma$  level [54] which is not modified by hypoglycemic treatment [55].

### 3.3. Crosstalk between adipocytes and endothelial cells

Vascular ECs play a major role in maintaining cardiovascular homeostasis. In addition to providing a physical barrier between the vessel wall and blood lumen, endothelium secretes a number of mediators that regulate vascular tone, coagulation, fibrinolysis and blood cells trafficking. Endothelium can extend its repertoire of functions by adaptation to various stimuli, including mechanical stress, oxidative and metabolic stress, inflammation, hypoxia and many others [32].

Obesity is a component of a metabolic syndrome, a constellation of metabolic risk factors that consist of (i) dyslipidemia, (ii) hypertension, (iii) glucose intolerance, (iv) IR, (v) prothrombotic and (vi) a pro-inflammatory state. Hyperglycemia, dyslipidemia, hyperinsulinemia and adipokines derived from AT play a more dominant role in microvascular complications. In addition to the endothelial pro-inflammatory activation and the decrease in NO production, endothelial barrier increases its permeability due to increased VEGF synthesis in response to hypoxia (HIF-1 activation) and the presence of FFAs released from adipose tissue as an effect of insulin resistance (**Figure 6**) [56]. The strong interaction between AT pro-inflammatory adipokines and endothelium makes obese patients much more prone to CVD [2]. Hanzu et al. exposed endothelium on the medium supplemented with extracts obtained from the visceral fat taken from obese and lean subjects. The adipokines secreted from the visceral fat taken from the obese adversely affected endothelium by increasing the expression of adhesion molecules and von Willebrand factor (vWF). That, in turn, intensified the endothelial cell proliferation and changed EC morphology. Researchers concluded that the observed effects are a result of the activation of NF- $\kappa$ B transcription factor signaling pathways [57].

Endothelial dysfunction in obesity is a multifactorial process and has different molecular aspects. Obesity is characterized by an increased generation of ROS. Because of endoplasmic reticulum stress and mitochondrial dysfunction, ROS are generated in the vascular wall and hypertrophied adipocytes. The effect of ROS on vascular function critically depends on their



**Figure 6.** Crosstalk between adipocytes and endothelial cells [3, 4, 58]. *Abbreviations:* IL-6, interleukin 6; TNF-alpha, tumor necrosis factor-alpha; NF-κB, transcriptional factor; HIF-1α, transcriptional factor; FFA, free fatty acid; NO, nitric oxide; VEGF, vascular endothelial growth factor.

quantity. When formed in low amounts, they can act as intracellular secondary messengers, modulating the growth response of vascular smooth muscle cells and fibroblasts. A higher amount of ROS can cause widespread cellular toxicity. Many enzymes of the mitochondrial electron transport chain, such as COX (cyclooxygenase), LOX (lipooxygenase), xanthin oxidase, myeloperoxidase, NADPH oxidase, uncoupling eNOS and leptin are the major contributors of ROS production in obesity, leading to a decrease in NO production and an increased production of vasoconstrictor ET-1. NO bioavailability is lowered as a result of peroxynitrite formation ( $\text{ONOO}^-$ ). Peroxynitrite is also created as a result of the iNOS activity which is stimulated by an exaggerated production of TNF- $\alpha$ . The enzyme produces NO in a large amount and when combined with the superoxide ( $\text{O}_2^-$ ), anion creates cytotoxic peroxynitrite. Finally, NO production can also be inhibited by an endogenous inhibitor— asymmetric dimethylarginine (ADMA), which competitively inhibits eNOS. The ADMA level is elevated in obese patients and could serve as another mechanism which alters the NO level [4, 32].

ROS accumulation and pro-inflammatory adipokines are implicated in the activation of NF-κB, which is involved in the immune response, apoptosis and inflammation regulating the expression of growth factors, pro-inflammatory cytokines and adhesion molecules [31]. Many products of the genes regulated by NF-κB also, in turn, activate NF-κB (e.g. TNF- $\alpha$ ). Pro-inflammatory mediators created by NF-κB signaling, and derived from AT are implicated in EC activation with an increased expression of adhesion molecules (ICAM-1—intercellular cell adhesion molecule-1, VCAM-1—vascular cell adhesion molecule and selectins), and an increased production of chemotactic factors (MCP-1 and IL-8). This promotes the adhesion and migration of circulating leukocytes, initiating atherosclerotic lesion [32, 56].

ECs use glucose and FFAs as nutrients. Non-esterified FFAs are liberated from triglyceride-rich lipoproteins by the endothelial lipoprotein lipase. The endothelial glucose uptake is insulin

independent. Physiologically, glucose uptake in endothelium occurs via the glucose transporter GLUT-1. The insulin receptor is presented on the EC surface. Insulin can dilate arteries by the PI3K-Akt-eNOS signaling pathway that stimulates NO release and is also able to rapidly release ET-1 (via MEK-ERK1/2-ET-1 pathway). Both effects occur via the insulin receptor [56]. Central obesity is associated with an increased FFAs level. Elevated FFAs may impair endothelial function as measured by flow-mediated dilatation (FMD) and might affect insulin-mediated vasodilatation [56]. FFAs alter some important intracellular pathways: they could affect ion transport ( $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$ ), vascular reactivity (PKC—protein kinase C) cell growth and ROS generation (NADPH oxidase). This action may have potentially relevant implications for obese patients, leading to a decrease in NO bioavailability. Another possible mechanism, induced by elevated FFAs, that could impair vasodilatation in obese patients, is the reduction of prostacyclin ( $\text{PGI}_2$ ) production [32, 56].

The most essential adipokines implicated in EC dysfunction are leptin and adiponectin (Tables 1 and 2). Their specific properties affecting endothelium and angiogenesis processes are described below.

Leptin is secreted from WAT in proportion to the size of AT. It exerts a pressor effect by activating the central nervous system, which inhibits appetite. Its adverse multidirectional effects exerted on ECs include: (i) promoting oxidative stress, (ii) promoting thrombosis by inhibiting thrombomodulin level and increasing tissue factor, (iii) stimulating angiogenesis by promoting ECs proliferation and expression of adhesion molecules, MMPs and VEGF and (iv) stimulating pro-inflammatory cytokines such as  $\text{TNF-}\alpha$ ,  $\text{Il-6}$  and  $\text{MCP-1}$  [4, 59]. This stimulating effect of pro-inflammatory cytokines is responsible for ECs activation, and may cause hypertension. However, it has been recently shown that leptin may also have a vasodilatory effect. This heterogeneous effect relates to the predominant role of the endothelium-derived hyperpolarizing factor (EDHF) mechanism and is induced by a direct effect of NO release from ECs and an indirect effect of NO release from adipocytes, which triggered leptin, activates eNOS [60].

Adiponectin is the most abundantly secreted adipokine (plasma concentration: 2–20  $\mu\text{g/ml}$ ). Globular adiponectin (gAD) and full-length adiponectin (fAD) exert their effect by two receptors (Adipo R1 and Adipo R2). Both receptors are presented on ECs. Generally, adiponectin is responsible for insulin sensitivity by improving carbohydrate and lipid metabolism. Adiponectin exerts its insulin-sensitizing effect by increasing  $\beta$ -oxidation of FFAs, reducing serum triglyceride and FFAs. It also has antiatherogenic and anti-inflammatory properties. The production of adiponectin by adipocytes is inhibited by pro-inflammatory factors such as  $\text{TNF-}\alpha$  and  $\text{Il-6}$  as well as hypoxia and oxidative stress. Its antiatherogenic and anti-inflammatory properties within the vascular wall are mediated via: (i) increased phosphorylation of insulin receptor, (ii) modulation of  $\text{NF-}\kappa\text{B}$  pathway (inhibiting adhesion molecules), (iii) inhibition of foam cell formation, (iv) decreased proliferation and migration of smooth muscle cells and (v) stimulation of NO production in ECs. The plasma adiponectin level highly correlates with the vasodilatory response. Conversely, hypoadiponectinemia is associated with a blunted endothelial function and coronary artery disease [3, 12]. Adiponectin can also induce angiogenesis by promoting signaling cross talk (AMPK-Akt-eNOS) in endothelium. Interestingly, a

potent inhibition of endothelial angiogenic properties like proliferation and migration was also observed [61, 62].

The major risk factors for coronary artery disease, present in obese patients, impair the endothelium response to acetylcholine (ACh), which induces a paradoxical vasoconstriction rather than vasodilatation [32]. The endothelial damage can also be assessed by measuring some endothelial-derived markers. Hemostatic factors such as procoagulant von Willebrand factor and anticoagulant TM are elevated in obesity. They are not only the markers of EC activation but also the markers of EC membrane injury. The factors responsible for EC activation, which mediate the interaction between leukocytes, platelets and the endothelium, are also elevated in obese patients (E-selectin, VCAM-1 and ICAM-1). These factors provide potentially relevant information about the EC condition and the tendency to vasoconstriction, coagulation, platelet aggregation and future cardiovascular morbidity and mortality [4, 32].

#### 4. Caloric restriction

Caloric restriction (CR) is the most effective and reproducible dietary intervention known to affect aging process and increase the healthy lifespan in various model organisms from unicellular yeast to rodents and primates. There is no agreement on how severe a CR must be in order to confer benefits in different organs and systems. However, CR which in most cases involves a 20–40% reduction of dietary requirement relative to normal intake is a severe intervention that results in both beneficial and detrimental effects [63, 64]. Studies show that CR does not need to be prolonged for a long time to be effective, with the advantage that short-term CR is easier to include in clinical practice. In this context, a genomic analysis revealed that the results obtained from short- and long-term CR were similar [65]. It is one of the most common and cost-effective interventions used to induce body weight reduction and control CVD risk factors. It is important to note that the induction of negative energy balance is mandatory for achieving the metabolic benefits of weight loss. Benefits on CV risk factors by reducing the daily caloric intake have been widely described in obese subjects [7, 65–67]. CR reduces body weight, waist circumferences (visceral fat), serum lipids, insulin level and improves insulin sensitivity. The decrease in adiposity leads to a reduction of pro-inflammatory adipokines (e.g. leptin, Il-6, TNF- $\alpha$ , etc.), oxidative stress as well as to an increase in the anti-inflammatory adipokines (e.g. adiponectin, omentin, etc.) [7, 66–68]. Weight loss enhances FMD, which significantly improves endothelial function *in vitro* [8].

The molecular mechanism of CR is complex. It involves downregulation of insulin (also IGF-1 pathway) and insulin-like signaling, the signaling of mTOR (mammalian target of rapamycin) kinase pathway, a rise in the energy balance modulator sirtuins (particularly sirtuin 1) as well as a decrease in pro-inflammatory mediators, growth factors and ROS production [63]. Especially sirtuins are responsible for some beneficial and longevity-promoting effects of CR in many species of animals—from fruit flies to mammals. They are implicated in many physiological effects as control of circadian clock, mitochondrial biogenesis, aging, apoptosis and inflammation [69].

Large observational data support a detrimental effect of obesity on the risk of several cancers, including breast and colon cancer, two of the most common cancers in North America and Europe [63]. The most important causes predisposing to cancer development in obese people are elevated

female sex hormones, hyperinsulinemia and a high level of pro-angiogenic and pro-inflammatory factors. Relatively little data exist on the effects of weight gain or weight loss on the risk of cancers [63]. The lack of data on weight loss is likely a function of the small number of individuals able to achieve a sustained weight loss. It is relatively often emphasized that the risk of colorectal cancer is reduced due to weight loss [70]. The best evidence that weight loss can reduce the risk of cancer comes from recent studies in bariatric surgery patients [71]. Tumors become malignant when they attract new blood vessels. Angiogenic switch could be slowed down when special drugs which can stop a key angiogenic mediator—VEGF are used. This concept of angiogenesis was first described by the pediatric surgeon Folkman [72]. The balance between pro- and anti-angiogenic factors allows neoangiogenesis to occur. Angiogenesis could be inhibited through an action on VEGF, bFGF and MMPs. Additionally, high level of mitogenic insulin resistance (IR) correlates with some angiogenic factors [73]. It is well documented that pro-inflammatory cytokines in obesity are mitogenic and pro-angiogenic. CR can decrease (i) insulin signaling, (ii) angiogenic mediators, (iii) inflammation lowering pro-inflammatory adipokines, NF- $\kappa$ B signaling and COX-2 expression, (iv) pro-angiogenic leptin and (v) increase anti-angiogenic adiponectin [74–76]. This anti-inflammatory effect of CR contributes significantly to crucial endothelial function in regulating angiogenesis, hemostasis, vascular tone and vascular wall integrity. This modified effect of CR exerted on endothelium is not only caused by decreased inflammation and angiogenic factors, but also by regulating fibrinolysis, the integrity of the basement membrane and extracellular matrix proteins [20]. Plasminogen activator inhibitor-1 (PAI-1), t-PA, u-PA and also MMPs are involved in angiogenesis. The circulating levels of PAI-1 and MMPs are consistently decreased in response to CR [4]. Rats fed a diet reduced by 40% showed improved vascular EC function, reduced free radical production, expression of NF- $\kappa$ B and a decreased expression of pro-inflammatory genes such as IL-6, TNF- $\alpha$ , sICAM-1 or iNOS [77]. Furthermore, the positive effect of a reduced caloric intake leads to an increased expression of eNOS and transcriptional factor Nrf2 (nuclear factor erythroid 2-related factor), which produces anti-oxidative stress proteins, and activates the VEGF-dependent metabolic pathways [64, 74, 77].

## 5. Effects of moderate caloric restriction on obese patients—personal observations

In the next section, we would like to present the data of our studies where we investigated the effect of moderate CR on: (i) endothelial cell function especially involved in angiogenesis, (ii) production of adipokines, angiogenic and angiostatic factor and (iii) oxidative stress. Based on our previous studies that have already been published [78] and the data from the literature, we hypothesized that moderate CR, because it is not so burdensome and reflect a real-life situation, seems to be optimal to achieve an improvement of EC.

### 5.1. Patients and experimental design

To assess the impact of moderate CR, we recruited 50 obese patients (age  $37 \pm 11$  years, BMI:  $37.7 \pm 6.1$  kg/m<sup>2</sup>, 72% women). The study was approved by the institutional Ethics Committee (decision number: 217/11) and all patients submitted their informed consent. The exclusion criteria involved overt diabetes, congestive heart failure, an acute coronary syndrome over the



past 6 months, malignant or systemic illness, pregnancy, bariatric surgery, a known eating disorder and a change in body weight greater than 2 kg over the past 3 months. Glucose intolerance and hypertension are very common abnormalities seen in obese patients; therefore we decided to include them in our study. Glucose intolerance was the most common disorder (46%). Therefore, the obese patients were divided into the normoglycemic (N) obese (age  $37 \pm 12$  years, BMI:  $36.2 \pm 6.1$  kg/m<sup>2</sup>, 78% women); treated only with a diet (n = 27) and the obese with glucose intolerance (GI) (age  $38 \pm 10$  years, BMI:  $38.8 \pm 7.6$  kg/m<sup>2</sup>, 70% women) and treated with a diet and a hypoglycemic drug metformin (n = 23). The results were derived from all 50 obese patients who completed the 8-week mild CR program and as a result of this intervention they reduced their body weight.

The dietary intervention lasted 8 weeks and aimed to produce a 15–30% energy deficit (a reduction by 300–500 kcal/day). The patients' basal metabolic rate (BMR) was calculated according to the Harris-Benedict equation and corrected for physical activity according to WHO criteria [79]. The estimated BMR ranged between 1454 and 2045 kcal/d [79] and all patients displayed low physical activity (physical activity factor: 1.4) [80]. The participants were supervised twice a week by a dietician, who designed individualized dietary plans that supplied energy from similar sources but took into account patients' food preferences. The diet was composed of: 25% fat: saturated 7%; 20–25% protein; 50–55% carbohydrates: complex 45–50%, saccharose <10% (exemplary diet is presented in appendix). To assess only the effect of mild CR, physical activity was not recommended.

## 5.2. Detected parameters

To minimize diurnal variations, fasting blood samples were always collected between 7.30 and 9.00 am. Samples of serum were aliquoted and stored at  $-80^{\circ}\text{C}$  until assayed. Before and after CR we measured the following parameters:

- mediators of ECs function (sICAM-1, sVCAM-1, sE-selectin, TM, vWF, PAI-1, ADMA, NO);
- proliferation, migration and invasion using endothelial cell culture *in vitro* after exposition to medium supplemented with 20% serum taken before and after CR;
- adipokines (leptin, adiponectin, vaspin, resistin, TNF- $\alpha$ , Il-6);
- angiogenic factors (VEGF, bFGF, Ang-1, IGF-1, Il-8, MMP-2, MMP-9);
- angiostatic factors (Ang-2, endostatin, TSB-1, TIMP-1, INF- $\gamma$ , IP-10);
- oxidative stress (TAS—total antioxidant status, SOD—superoxide dismutase, catalase, ROS production by ECs *in vitro*);
- fat content;
- homeostatic model of assessment of insulin resistance (HOMA-IR).

## 5.3. Methods

Using the *in vitro* culture (HUVEC line EA.hy926), we evaluated endothelial pro-angiogenic processes, such as proliferation, migration and invasion.

- Cell proliferation was measured using an MTT assay (methylthiazol tetrazolium assay) [81]. Briefly, monolayers of  $2 \times 10^4$  ECs were exposed to standard medium (M199, Sigma, USA) supplemented with 20% serum taken before and after CR for 24 h in hypoxic condition (1% O<sub>2</sub>). After the exposition, cells were incubated in a medium containing 1.25 mg/ml of the MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) for 4 h at 37°C. The active mitochondrial dehydrogenases metabolized the conversion of MTT salt [82]. The generated formazan product was dissolved with the acidic solution of sodium dodecyl sulfate and N,N-dimethylformamide. Absorbance of the converted dye was recorded at 595 nm with a reference wavelength of 690 nm.
- Migration and invasion were tested using Boyden chamber (Cultrex Kit, USA). Briefly, ECs were grown to 80% confluency in a culture medium. Then the cells were harvested, resuspended in serum-free medium and placed in an upper migration chamber ( $5 \times 10^4$  cell/100  $\mu$ l). To detect cell migration, this chamber was coated only with assay buffer in the contrary to invasion process where this surface was coated with basement membrane extracts. Cells were then stimulated for 24 h in hypoxic condition (1% O<sub>2</sub>) with standard medium supplemented with 20% serum taken before and after CR placed in the lower chamber. Migrated cells were detached and treated with calcein AM in the lysis buffer. Fluorescence of cell lysates was measured using 480 and 520 nm wavelengths for excitation and emission, respectively.
- Generation of ROS by endothelial cells treated with standard medium supplemented with 20% serum taken before and after CR for 24 h was assessed by labeling with 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA, Molecular Probes, USA) that is trapped inside the cells and activated by intracellular ROS. Briefly, following the exposure to medium,  $2 \times 10^4$  cells were loaded with 10  $\mu$ M H<sub>2</sub>DCFDA for 30 min and then treated with the lysis buffer. Fluorescence emitted by cell lysates was measured using wavelengths of 485 and 535 nm for excitation and emission, respectively [83].
- To detect serum factors we used the immunoassays from R&D Systems (USA).
- Nitric oxide and TAS were measured by colorimetric assays from R&D Systems (USA) and Cayman (USA), respectively.
- SOD and catalase were tested using enzymatic tests from Cayman (USA).
- Fat content was estimated by bioelectrical impedance analysis (Tanita/Acern, Japan).
- Homeostasis model assessment (HOMA-IR)—an index of insulin resistance was measured using the following equation: fasting insulinemia (mU/ml)  $\times$  fasting glycemia mg/dl/405 [84].

#### 5.4. Statistical analysis

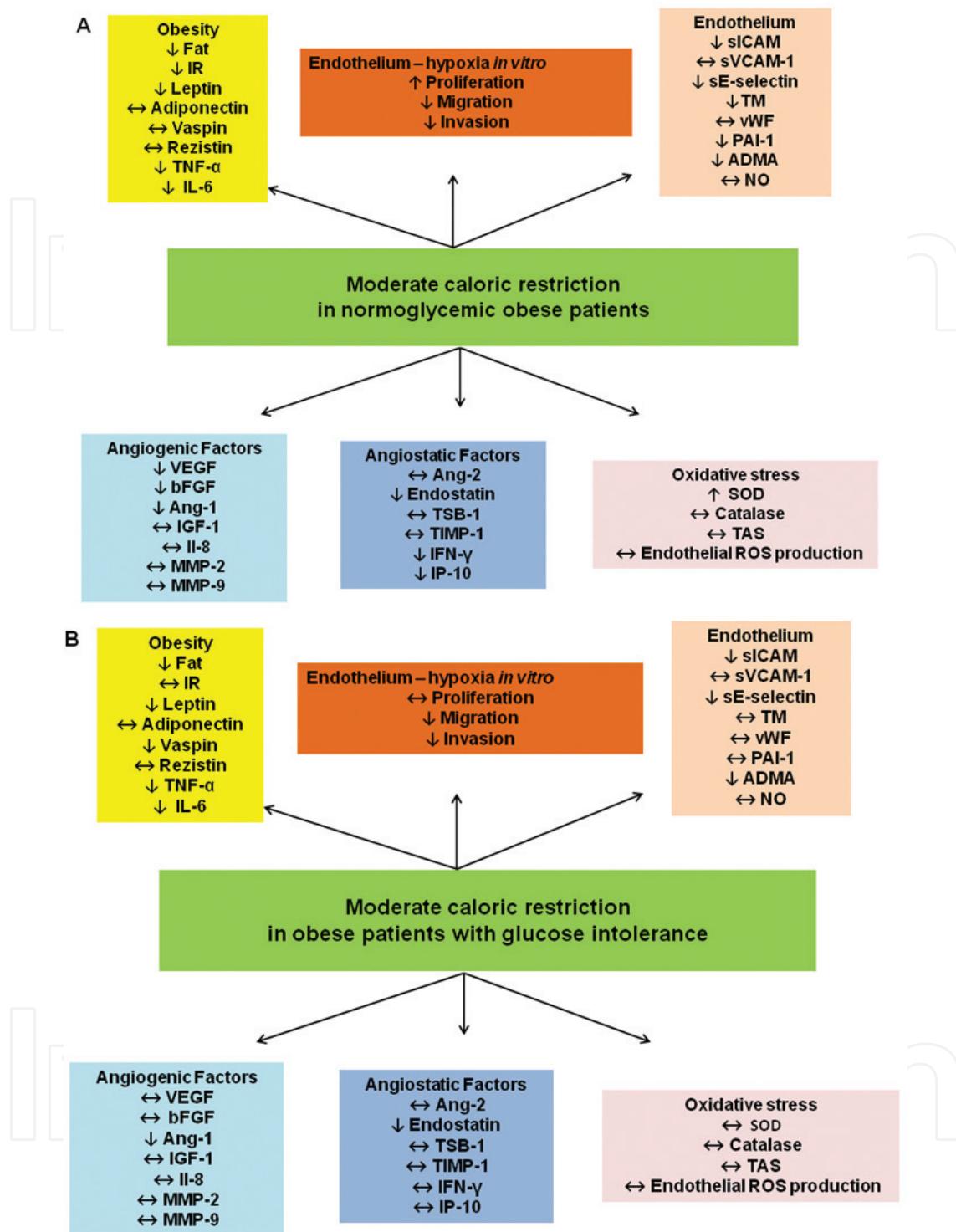
Statistical analysis was performed using GraphPad Prism™ 6.00 (GraphPad Software Inc., San Diego, California). The Wilcoxon test and the Mann-Whitney test were used for comparing paired and unpaired data, respectively. The data were also analyzed with repeated measures analysis of variance using a post hoc test for multiple comparisons. Associations between variables were assessed with the Spearman correlation. The level of significance was set at  $p < 0.05$ .

## 5.5. Results

Moderate CR induced a reduction of the anthropometric measurements and angiogenic adipokines in all subjects (leptin, II-6 and TNF- $\alpha$ ) (**Figure 7A and B**). The largest decrease was achieved in TNF- $\alpha$  concentration in normoglycemic obese patients (N:  $-66 \pm 5\%$  vs. GI:  $-38 \pm 7\%$ ). Similarly, a decrease in fat mass was greater in obese patients with a normal glucose profile (N:  $-10.4 \pm 2.1\%$  vs. GI:  $-8.7 \pm 3.3\%$ ). CR also decreased the percentage of patients with life-threatening obesity from 34 to 18%. Actually, more beneficial changes in lipids and carbohydrates parameters were observed in normoglycemic obese subjects (HOMA-IR: N:  $-27 \pm 4\%$  vs. GI:  $-8 \pm 2\%$ ; cholesterol: N:  $-9 \pm 5\%$  vs. GI:  $-1.5 \pm 1\%$ ; triglycerides: N:  $-24 \pm 9\%$  vs. GI:  $-7 \pm 4\%$ ). CR was less effective in the obese with GI, certainly because of a higher percentage of patients with life-threatening obesity (GI: 52% vs. N: 34%). Dietary treatment significantly reduced the pro-angiogenic (VEGF:  $-11 \pm 6\%$ , bFGF:  $-35 \pm 10\%$ , Ang-1:  $-18 \pm 9\%$ ) and angiostatic (endostatin:  $-126 \pm 5\%$ , IP-10:  $-76 \pm 14\%$ , IFN-gamma:  $-74 \pm 17\%$ ) factors, especially in normoglycemic patients (**Figure 7A and B**). In the obese with GI, CR reduced only two angiogenic parameters of 13 analyzed (angiostatin-1:  $-27 \pm 7\%$ , endostatin:  $-8 \pm 2\%$ ). This group was also characterized by a higher concentration of VEGF ( $+105 \pm 12\%$ ), IFN-gamma ( $+225 \pm 24\%$ ), IP-10 ( $+103 \pm 25\%$ ) and lower IGF-1 ( $-49 \pm 15\%$ ) after the treatment when compared to the normoglycemic obese. It should be emphasized that, at baseline, the GI group was characterized by a higher concentration of VEGF ( $+93 \pm 18\%$ ) and lower IP-10 ( $-45 \pm 13\%$ ). Additionally, the decrease in pro-angiogenic leptin and bFGF was positively correlated with the reduction of anthropometric measurements (body mass, BMI, WC (waist circumference) and fat mass) after dietary intervention. CR in both tested groups, in a comparable way, reduced pro-inflammatory markers of endothelial activation (sICAM-1 in both groups  $-5 \pm 1.5\%$ ; sE-selectin: N:  $-21 \pm 4\%$  vs. GI:  $-42 \pm 10\%$ ) and ADMA (N:  $-35 \pm 5\%$  vs. GI:  $-37 \pm 10\%$ ), but did not change the production of NO (**Figure 7A and B**). The changes in coagulation and fibrinolysis parameters were far less pronounced especially in obese patients with GI. Mild CR was only partially effective in reducing oxidative stress by increasing SOD in obese normoglycemic patients. The culture medium supplemented with serum obtained from obese patients, before and after CR, modified endothelial function essential for angiogenesis. We have documented an increase in endothelial proliferation and a decrease in endothelial migration and invasion after 8 weeks of CR under hypoxic condition. These observations were less pronounced in the obese with GI (**Figure 7A and B**).

## 5.6. Discussion

Eight weeks of moderate CR reduced the anthropometric measurements (BMI, body weight and fat mass), pro-angiogenic and pro-inflammatory adipokines such leptin, II-6 and TNF- $\alpha$  in all obese patients. Additionally, in both tested groups (i.e. in normoglycemic and in glucose intolerance participants), CR in a comparable way, reduced pro-inflammatory markers of endothelial activation (sICAM-1 and sE-selectin), inhibitor of eNOS—ADMA, but did not change the production of NO. Worth emphasizing is that more beneficial changes were observed in normoglycemic obese. We have observed: (i) the improvement of laboratory tests assessing carbohydrate and lipid profile (especially HOMA), (ii) reduced level of many angiogenic and angiostatic factors and (iii) modification of angiogenic properties of EC. Moderate



**Figure 7.** Effect of moderate caloric restriction in (A) obese normoglycemic patients, (B) obese patients with glucose intolerance. *Abbreviations:* IR, insulin resistance; Il-6, interleukin 6; TNF-alpha, tumor necrosis factor-alpha; sICAM-1, soluble form of intercellular cell adhesion molecule-1; sVCAM-1, soluble form of vascular cell adhesion molecule-1; sE-selectin, soluble form of selectin E; TM, thrombomodulin; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; ADMA, asymmetric dimethylarginine; NO, nitric oxide; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; Ang-1, angiotensin 1; IGF-1, insulin-like growth factor 1; Il-8, interleukin 8; MMP, metalloproteinase; Ang-2, angiotensin 2; TBS-1, thrombospondin-1; TIMP, tissue inhibitor of metalloproteinase; INF-γ, interferon γ; IP-10, interferon-inducible protein; ↔, without changes.

CR has probably not exerted as many beneficial effects in the obese patients with GI because this group was characterized by a greater number of patients with life-threatening obesity. The parameters of oxidative stress were the least susceptible for modification by moderate CR. Additionally, we have documented a positive correlation with the reduction of all tested anthropometric measurements after dietary intervention and a decrease in pro-angiogenic leptin and bFGF.

Weight reduction in obese people is not easy to achieve due to the difficulty in maintaining a dietary regimen and the usual co-existence of IR. Insulin resistance makes it harder for patients to lose unnecessary body weight by hindering glucose utilization by the muscles and liver [85]. There is no doubt that CR improves endothelial function [7–9, 68], particularly the concentration of adhesive molecules, pro-inflammatory cytokines and NO production [7, 8, 66]. It is well documented that changes in NO production by endothelium is triggered by diet are generally related to changes in weight loss [7, 8, 67], plasma glucose concentration [68] and duration of CR [86]. However, a short-term dietary intervention does not always improve vascular endothelial-derived NO response [87, 88]. The parameters involved in coagulation and fibrinolysis are less prone to modification [7, 66, 67].

An important issue is whether even a small degree of CR, resulting in a modest loss of body weight, will improve endothelial function. To investigate this, it is necessary to find a parameter that is sensitive enough to reflect an improvement of endothelial function even with only a slight weight loss. Using ECs cultured *in vitro* in medium supplemented with serum taken from the obese patients before and after CR, we found a correlation between EC proliferation and weight loss after CR. This effect was especially apparent in male subjects [78]. The mechanisms underlying changes in EC angiogenic properties in response to dietary intervention are difficult to define unequivocally. They are probably context-dependent. In addition to the effects exerted by leptin and adiponectin through their similar receptors [59] and the effect of sex hormones [89, 90], changes in EC metabolism may be exerted by the alteration of energy homeostasis [91, 92]. Various metabolic pathways are now recognized as contributing significantly to obesity-associated angiogenesis [93, 94]. Proliferation is an energy-consuming process, it is tempting to hypothesize that the magnitude of serum-induced endothelial growth response reflects a tendency for conserving energy during CR. Interesting observations have recently been published by Reinhardt et al. [91, 92]. They observed that patients with a “thrifty” phenotype (economic and energy saving) could distribute more energy for cell proliferation and lose less weight during CR, while patients with a “spendthrift” phenotype (wasteful and energy spending) would spend less energy for the cell proliferation and lost more weight after CR [91]. Accordingly, we observed that individuals who had lost more weight exhibited a decrease in cell proliferation (for quantitative data see [78]). We documented that a moderate CR in obese subjects changes the endothelial genes expression profile involved in the cell cycle [78]. Similarly, Ellsworth et al. have recently revealed significant changes in peripheral blood gene expression patterns, including those involved in cell cycle in obese patients undergoing intensive long-term lifestyle modifications. Observed changes occurred only in patients who achieved considerable weight loss (>10%) over 1 year, but not in participants with minimal weight loss [65]. As the authors emphasized, the mechanism by which a CR protects the function of capillaries remains unexplained. It certainly

improves many of the endothelial functions essential for angiogenesis, such as proliferation, tube formation and prevents against apoptosis and aging [74], Csiszar et al. explained that the reduction of oxidative stress and inflammation would primarily improve EC functions. Dietary intervention drives a change in the concentration of many neuroendocrine factors, which reach the capillary ECs from the bloodstream and initiate a variety of cytoprotective processes [77].

We have measured 13 factors involved in angiogenesis (angiogenic/angiostatic). Reducing AT after the dietary treatment makes it less demanding for the factors necessary for angiogenesis. Dietary restriction led to a decrease in the concentration of three pro-angiogenic factors (VEGF, bFGF and Ang-1) and three angiostatic factors (endostatin, IP-10 and IFN- $\gamma$ ) in normoglycemic obese subjects. In obese patients with GI, CR reduced only 2 parameters involved in the angiogenesis process, out of 13 analyzed (Ang-1, endostatin). Glucose intolerance in obese people adversely affects the angiogenesis process. This has been confirmed by Nathan et al. where the lower adhesion, migration and tubular structure formation in endothelium were observed compared to the normoglycemic control group [95].

One of the most important factors in the angiogenesis process that stimulates migration and EC proliferation is VEGF [29]. Miyazawa-Hoshimoto et al. have demonstrated a positive correlation between serum VEGF levels and anthropometric parameters of obese persons, which indicates that visceral fat is the most important factor that determines the VEGF concentration in obesity. We have also observed that obese patients with high BMI and fat mass (particularly obese with GI) exhibit elevated VEGF level at baseline when compared with normoglycemic obese; nevertheless, CR did not reduce the level of VEGF. Weight reduction might decrease VEGF concentration [96], however, this effect is not always achieved [52, 75, 97]. We have observed a decline in VEGF level only in the normoglycemic obese. Higher VEGF level is characteristic for the obese with GI when compared with the normoglycemic patients [52, 98]. Insulin stimulates VEGF production in vascular ECs [99] and in adipocytes [100] by stimulating the HIF-1 $\alpha$  expression [29]. The authors emphasize that insulin is a potent mitogen, and its stimulatory effect on VEGF production and proliferation is already present at physiological concentrations [29, 100]. EC proliferation after CR was higher in the normoglycemic obese and was not observed in patients with GI despite significantly higher insulin and VEGF concentrations. Severe obese patients with glucose intolerance treated with metformin and/or moderate CR not always reduced insulin concentration and HOMA levels [101, 102]. EC proliferation in obese subjects is complex and cannot be explained by the effects of typical angiogenic factors as elevated level of insulin and VEGF [78]. Yamagishi et al. performed an experiment showing that, despite higher VEGF level following insulin stimulation, no increase in VEGF receptor-mediated EC proliferation was observed. They concluded that this effect may hamper the response to pro-angiogenic VEGF in patients with hyperinsulinemia [99]. Recent work by Aplin and Nicosia also confirms the decline in expression of VEGF receptors in the EC under hypoxia [30]. Experiment done by Csiszar et al. using nonhuman primate *Macaca mulatta* after 10 years of CR showed similar observation [74].

bFGF is the subsequent crucial angiogenic factor modified by weight loss [78, 96]. The correlation between bFGF and abdominal obesity is obscure [40, 41], nevertheless we observed a positive correlation between the decrease in bFGF and the reduction in body mass, fat mass,

BMI and waist circumference. bFGF changes endothelial angiogenic properties [38]. The correlation between bFGF and MMPs in an endothelial culture medium suggests that the expression of MMPs is critical for the migration and invasiveness of cells in the formation of new blood vessels [39]. The significant lowering of bFGF in patients treated with a diet alone was probably one of the most important factors that contributed to the decreased migration and invasiveness of EC after the intervention.

Endostatin inhibits the proliferation, migration, adhesion and ability to form the tubes by altering the action of VEGF and bFGF. It blocks multiple signaling pathways (TNF- $\alpha$ , NF- $\kappa$ B, adhesion and clotting) [47]. The elevated endostatin concentrations are characteristic of overweight patients [37]. Eight weeks of moderate CR was enough to decrease endostatin concentration in both obese groups. It is worth to emphasize that endostatin was the only angiostatic parameter modified by CR in obese patients with GI.

Ang-1 and Ang-2 control the maturation and stabilization of blood vessels [42, 48] and by that means regulate AT growth [48]. Dietary restrictions reduce their concentration [75]. Ang-1 was the only angiogenic parameter that was reduced in obese patients with GI. Since Ang-1 stimulates proliferation and migration, its reduced concentration after CR could also be responsible for diminished endothelial angiogenic function observed *in vitro*. Ang-2 is synthesized almost exclusively by ECs cells during vascular remodeling [103]. It destabilizes the vascular wall to facilitate the action of other pro-angiogenic factors [104]. The mechanism of angiotensins' action is not fully understood. Ang-2 has dual pro- and anti-angiogenic properties. It is believed that Ang-2 acts via a Tie-2 receptor as its antagonist, when Ang-1 is not available or acts independently without a Tie-2 receptor [105]. Higher level of Ang-2 is observed in patients with type 2 diabetes [106] as a hyperglycemia effect [107]. It has been suggested that elevated concentrations of Ang-2 and hyperglycemia may promote abnormal neovascularization and endothelial dysfunction, which in turn leads to diabetic micro- and macroangiopathy [108].

IP-10 is a chemotactic factor for T cells, produced by various cells such as monocytes, ECs, fibroblasts, in response to IFN- $\gamma$  stimulation [50]. Dalmas et al. show higher blood levels of IP-10 in obese patients, without any differences between diabetic and non-diabetic patients [52]. The group of obese patients with GI was characterized by a lower IP-10 (−45%) at baseline. CR reduced the IP-10 concentration by 76% only in the normoglycemic obese. The 10-year follow-up of patients with type 2 diabetes in the MONICA/KORA clinical trial suggests that the IP-10 protein is one of the risk factors for the clinical development of diabetes [109] and its concentration could be lowered by CR and lifestyle modification [110].

Infiltrating of immune cells in AT is an important factor leading to inflammation and IR [6]. Interferon- $\gamma$  is known to change the macrophage phenotype to more pro-inflammatory (M1) [53]. Patients with GI had a higher IFN- $\gamma$  concentration after CR. Higher fat content and stronger stimulation by macrophage-derived IFN- $\gamma$  were the important factors for higher concentrations of pro-inflammatory adipokines after the experiment (higher TNF- $\alpha$  after CR). Obese individuals usually have elevated IFN- $\gamma$  levels, particularly patients with central obesity [54]. Diet intervention significantly decreased IFN- $\gamma$  levels in the normoglycemic obese (−74%). Although plasma concentrations of IP-10 and IFN- $\gamma$  in the obese with GI after CR were significantly higher when compared with the normoglycemic obese, the diet did not change their concentrations. It is

well known that hyperglycemia in diabetic patients significantly modifies the immune response, particularly the humoral immunity [12]. Higher levels of IP-10 and IFN- $\gamma$  seen in the obese with GI, may reflect a different immune response observed in this group.

Metformin is one of the oldest commonly used oral hypoglycemic drugs, which does not affect insulin secretion. It functions omnidirectionally on various cells [85, 111], reducing the risk of CVD by improving blood vessels, vascular endothelium or decreasing inflammatory markers [85]. Animal models and *in vitro* experiments showed the anti-angiogenic effect of metformin and emphasized its beneficial role which goes far beyond lowering the glucose level [112, 113]. Endothelium treated with metformin alters the secretion profile of angiogenic and angiostatic factors [112]. Moreover, metformin protects the myocardium against hypoxia. The cardioprotective effect of metformin is the result of the reduced oxidative stress and bFGF level, which are responsible for hypertrophy and myocardial fibrosis [111]. The ability to reduce bFGF concentration is one of metformin's anti-tumor activities additional to its inhibitory effect on the migration and proliferation of both endothelial and tumor cells [112, 113]. Our obese patients treated with CR and metformin had tendency for higher anthropometric parameters (more patients with life-threatening obesity), additionally more patients had treated hypertension. The angiogenic mediators and endothelial cell function were significantly less modified by moderate CR in compare with normoglycemic patients treated only with diet.

## 6. Conclusions

AT remodeling is pathologically accelerated in an obese state due to local hypoxia leading to reduced angiogenesis, severe immune cell infiltration with subsequent pro-inflammatory responses and additional deterioration of EC functions. It is believed that EC dysfunction in obesity can be reduced by CR. Moderate CR reflects a real-life situation and could be optimal to achieve an improvement in EC. Our observations suggest that a moderate CR can improve several parameters of EC function, especially those involved in angiogenesis. It also improves anthropometric and metabolic measurements, but does not significantly strengthen the antioxidant status. The *in vitro* model shows how various circulating factors, induced by CR, affect the endothelial proliferation, migration and invasiveness. This process is a result of a reduction of inflammation and a modification of angiogenic and angiostatic factors. Additionally, in patients with glucose intolerance, it is also caused by potential anti-angiogenic properties of metformin. The obtained results are particularly pronounced in the normoglycemic obese, and to a lesser extent in the obese with GI and IR, who may have an adverse impact on AT remodeling, the cardiovascular system and might have an increased risk of obesity-associated cancer diseases.

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## Conflict of interest

The author do not declare the conflict of interest.

## A. Appendix and nomenclature: Exemplary diet

### Diet plan

The energy supply was set at 1500 kcal

Meal	Hours	Share of energy supply (%)	Caloric value (kcal)
I breakfast	7:00–8:00	20	300
II breakfast	10:00–11:00	20	300
Lunch	13:00–14:00	10	150
Snack	16:00–17:00	30	450
Dinner	18:30–19:30	20	300

### Exemplary menu 1500 kcal—version 1

#### I breakfast—330 kcal

##### Italian sandwich “Caprese”

- 1 wholemeal roll (80 g)
- 1 small tomato
- Cottage cheese (50 g)
- Fresh basil
- 2 teaspoons of olive oil (10 g)
- Black pepper



#### II breakfast—310 kcal

##### Sandwich with egg and radishes

- 2 slice of wholemeal bread (80 g)
- 1 teaspoon of butter (5 g)
- Lettuce

- 1 boiled egg
- A few radishes

**Tomato juice—small bottle (300 ml)**

Lunch—440 kcal

**Italian spaghetti—1 portion**

Spaghetti should be boiled *al dente*.



Ingredients for 3 portions:

- 250 g wholemeal pasta
- 1 cane of tomatoes or fresh tomatoes
- 1 chicken breast (200 g)
- 10 capers
- 10 black olives
- 1 small onion
- 2 cloves of garlic
- Olive oil (1 spoon)
- Fresh parsley (2 spoons)
- Pepper, basil

Snack—160 kcal

**Natural yogurt—2% fat (180–200 g)** with 1 spoon of wheat bran.

**Tangerines (2 medium size—150 g)**

Dinner—290 kcal

**Tuna salad with parsley sauce (1 portion)**

**1 slice of wholemeal bread (40 g)**



- Rucola salad
- 100 g cocktail tomatoes
- 1/3 red pepper
- Canned tuna in its own sauce (50 g—2 spoons)
- Natural yogurt 2% fat (75 g—3 spoons)

- Sunflower seeds (15 g—3 teaspoons)
- Parsley
- Lemon juice

### **Exemplary menu 1500 kcal—version 2**

I breakfast—330 kcal.

#### **Sandwich with ham and vegetables**

- 1 slice of ray bred (40 g)
- Butter (1 teaspoon)
- Lettuce
- 1 slice of chicken ham (20 g)
- Small tomato (100 g)
- 1–2 small cucumbers

#### **Natural yogurt 2% fat (400 g)**

II breakfast—280 kcal

#### **Cottage cheese**

- Cottage cheese—3% fat (150 g)
- A few radishes (chopped or grated)
- Sunflower seeds (1 spoon)

#### **1 slice of ray bread (40 g)**

Lunch—440 kcal.

#### **Asparagus cream soup—1 portion**



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#### Ingredients for 4 portions

- 1 bunch of green asparagus
  - 1 zucchini
  - 1 clove of garlic
  - 1 onion
  - 1 liter of bullion
  - Olive oil (2 spoons)
  - Pepper
  - 1–2 teaspoons of lemon juice
  - pine nuts/ almond flakes (4 teaspoons)
  - cream cheese or natural yoghurt (4 teaspoons)
-

**1 wholemeal roll (80 g) or 2 slices of rye bread with butter (1 teaspoon)**

**1 orange (350 g) or ½ pomelo**

Snack – 160 kcal

**Oatmeal with dried plums (49 g)**

Dinner – 290 kcal

**Salad with baked pepper and tomatoes**

- 1 green or yellow pepper baked in the oven and peeled
- 1 big tomato (200 g)
- 1 spoon of olive oil or sunflower oil
- 1 spoon of chopped lettuce
- 1 spoon of chopped celery leaves
- Pepper

**Sandwich with ham**

- 1 slice of ray bread (40 g)
- 2 slices of chicken ham (40 g)

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