Metabolic Syndrome Individuals With and Without Type 2 Diabetes Mellitus Present Generalized Vascular Dysfunction

Cross-Sectional Study

Guillaume Walther, Philippe Obert, Fred Dutheil, Robert Chapier, Bruno Lesourd, Geraldine Naughton, Daniel Courteix, Agnès Vinet

Objectives—The first objective of this study was to demonstrate differences within endothelial-dependent and endothelial-independent vasoreactivity in macro- and microcirculation beds among patients with metabolic syndrome (MetS) with and without type 2 diabetes mellitus (T2D) compared with healthy counterparts. The second objective was to determine relationships among the function of macro- and microvascular systems and abdominal adiposity, as well as inflammatory markers in the 3 groups.

Approach and Results—Cross-sectional analyses of 53 patients with MetS without T2D and 25 with T2D, as well as aged 40 years and sex-matched healthy controls included microvascular (cutaneous blood flow measured with laser Doppler flowmetry in response to iontophoresis of acetylcholine and sodium nitroprusside), and macrovascular reactivity (flow-mediated dilation and nitrate-mediated dilation) along with anthropometric measures, plasma glucose, and insulin and inflammatory markers. Compared with controls, MetS participants showed depressed endothelial function of both micro- and macrocirculation beds. T2D in patients with MetS revealed an exacerbated vascular smooth muscle dysfunction in micro- and macrocirculation compared with MetS without T2D. Indices of micro- and macrocirculation were predominantly inversely related to abdominal fat and inflammatory markers.

Conclusions—MetS was associated with endothelial-dependent and endothelial-independent dysfunction, affecting both the macro- and the microvascular systems. Participants with diabetes mellitus demonstrated the most severe smooth muscle dysfunction. The presence of central abdominal fat and systemic inflammation seems implicated in the pathogenesis of vascular dysfunctions in MetS. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.304591.)

Key Words: metabolic brain syndrome ▪ microcirculation

Metabolic syndrome (MetS) is a clustering of cardiovascular risk factors, including central obesity, impaired fasting glucose, dyslipidemia, and hypertension. It is a key phenotype leading to atherogenic and diabeticogenic profiles. Endothelial and vascular smooth muscle dysfunction are considered early abnormalities in the development of atherosclerotic process in cardiometabolic disease.1,2 The vascular endothelium plays a pivotal role in maintaining vascular protective conditions and controlling smooth muscle tone. Specifically, control is managed through the release of potent vasodilators, such as nitric oxide (NO). Investigation of endothelial-derived NO function in large human vessels, through the noninvasive technique of flow-mediated dilation (FMD), has been largely used to predict cardiovascular outcomes in patients at risk of developing cardiovascular diseases (CVDs). In this context, MetS has been well characterized by morphological and functional disorders within large-sized vessels.3-5 However, most of the arterial vasculature is the nutritive network of smaller vessels (arterioles and capillaries), known as resistance arteries. These resistance arteries have a dominant influence on local blood flow (BF) and are directly connected with other tissues. Also, resistance arteries are submitted to chemical and neurohormonal stimulation and exposed to the continuous effects of shear stress generated by the blood stream.6 Moreover, emerging evidence suggests that coronary microvascular disease may...
appear before macrovascular disease in cardiometabolic disease, highlighting the relevance of early detection and comprehensive investigation of both micro- and macrovascular dysfunction.7

In patients with type 2 diabetes mellitus (T2D), microvascular and macrovascular dysfunction frequently coexist and are thought to be mediated, in part by reduced NO bioavailability8–10 resulting in progressive tissue damage. Nevertheless, in addition to the endothelium, vascular smooth muscle needs to be considered as a potential cause of vascular dysfunction in T2D, as recently demonstrated in a meta-analysis.11 Data about MetS microcirculation function are scarce and conflicting. Skin microvascular abnormalities in response to ischemia have been observed in individuals with MetS.5,12 More comprehensive studies have also observed endothelium-dependent dysfunction at a cutaneous13,14 and subcutaneous levels.15 However, only 1 study14 has also reported smooth muscle cell dysfunction in the skin microcirculation. What remains unknown is whether the presence of diabetes mellitus is a confounder in micro- and macrovascular dysfunction of individuals with MetS. Studies to date have failed to include MetS individuals without T2D. Subsequently, it also remains unclear whether associated micro- and macrovascular dysfunction is present in MetS individuals in a prediabetic state (ie, without T2D).

The links between vascular pathophysiology and abdominal obesity-related inflammation are also an emerging interest. Specifically, MetS has been linked to visceral abdominal fat that is recognized as a passive fat storehouse with high capacities of adipokines synthesis, leading to proinflammatory and insulin-resistant states.17 The link could explain, at least in part, macro- and microcirculatory impairments reported in this population in cutaneous and in subcutaneous vascular beds.5,12–14,16 Nevertheless, to the best of our knowledge, whether abdominal obesity and inflammation are associated with both macro- and microcirculation NO-related pathway in patients with MetS remains unknown.

Therefore, the first aim of this study was to compare endothelial-dependent and endothelial-independent vasoreactivity within macro- and microcirculation beds among patients with MetS with (MetS-T2D) and without T2D (MetS-NT2D) compared with healthy counterparts. The second aim was to explore relationships among the function of macro- and microvascular systems and abdominal adiposity and inflammatory markers in the 3 groups.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Anthropometric and Metabolic Variables
Patients with MetS and controls were matched for age and sex distribution (Table 1). Smoking history and medication distribution did not differ between MetS groups. Compared with controls, MetS individuals presented with significantly higher body mass index, body mass components, systolic arterial pressure, waist circumference, fasting glucose, glycated hemoglobin, triglycerides, insulin and homeostatic model assessment of insulin resistance, whereas high-density lipoprotein cholesterol was lower in MetS. Similarly, all inflammatory markers were higher in MetS groups than in controls (Table 1). However, comparisons of the 2 MetS groups showed greater values for waist circumference, fasting glucose, glycated hemoglobin, insulin, homeostatic model assessment of insulin resistance, and active plasminogen activator inhibitor-1 in MetS-T2D than in MetS-NT2D.

Vascular Structure and Endothelium-Dependent and Endothelium-Independent Function
In the microcirculation, resting cutaneous BF (CBF) did not differ among the 3 groups. CBF peak and the relative increase (CBF %) was adjusted for maximal dilatation (SNP and NMD), MetS individuals showed lower endothelium-dependent dilation (acetylcholine and FMD) was observed more frequently in MetS individuals than in controls. Similar results were observed in CBF responses to sodium nitroprusside (SNP) iontophoresis in MetS groups and controls (Table 2), with the exception of the relative increase (Figure 1). Compared with MetS-NT2D, MetS-T2D had lower SNP-induced reactivity (CBF % and cutaneous vascular conductance %), without differences in acetylcholine responses (Table 2; Figure 1). Results from the vascular assessments in the brachial artery showed significantly lower FMD values in MetS than in controls. In contrast, resting brachial artery diameter, resting and peak BF, and resting and peak shear rate were similar among the 3 groups (Table 2; Figure 1). Normalization of FMD using the Δshear adjustments yielded similar values among groups. Lower nitrate-mediated dilation (NMD) of the brachial artery was observed more frequently in MetS individuals than in the healthy controls. However, within the MetS groups, an exacerbated NMD dysfunction was observed in MetS-T2D than in MetS-NT2D (Figure 1). When macro- and microcirculation endothelium-dependent dilation (acetylcholine and FMD) was adjusted for maximal dilation (SNP and NMD), MetS individuals showed lower endothelium-dependent dilation index in small and large vessels than controls, without any apparent effects of T2D (Figure 2).

Relationships Between Endothelium-Dependent and Endothelium-Independent Parameters and Clinical Data
Linear regression and correlation analyses were performed to examine the relationships between endothelium-dependent

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BF</td>
<td>blood flow</td>
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<tr>
<td>CBF</td>
<td>cutaneous blood flow</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>FMD</td>
<td>flow-mediated dilation</td>
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<tr>
<td>MetS</td>
<td>metabolic syndrome</td>
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<tr>
<td>NMD</td>
<td>nitrate-mediated dilation</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
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<tr>
<td>T2D</td>
<td>type 2 diabetes mellitus</td>
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and endothelium-independent dilation in macro- and microcirculation, as well as clinical and inflammatory parameters. A relationship between acetylcholine CBF increase (%) and brachial FMD (%) \( r = 0.51; P < 0.001 \) was observed and persisted when either acetylcholine iontophoresis CBF or peak acetylcholine cutaneous vascular conductance was used in the model. No correlation was observed between SNP transdermal iontophoresis and NMD \( P > 0.05 \). Table 3 presents other significant correlates of clinical data and endothelium-dependent and endothelium-independent dilation in macro- and macrocirculation. In multiple regression analyses, acetylcholine CBF increase, central fat and active plasminogen activator inhibitor-1 emerged as independent predictors of FMD, explaining 30% \( P < 0.0001 \) of observed variance. Central fat emerged as a unique independent predictor of the CBF responses to acetylcholine and SNP iontophoresis, explaining, respectively, 17% \( P < 0.0001 \) and 8% \( P = 0.003 \) of observed variance. No variables were retained.
isolated arteries investigation. Skin microcirculation has, and have also produced heterogeneous responses to ex vivo differentially in vascular beds. Specifically, endothelial cells used to assess vascular function and is a demonstrated pre-

require less elaborate equipment than assessments involving

peripheral vascular beds represent convenient access and

Microcirculation

<table>
<thead>
<tr>
<th>Microcirculation</th>
<th>Controls, n=40</th>
<th>MetS-NT2D, n=53</th>
<th>MetS-T2D, n=25</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting CBF, PU</td>
<td>11.1±7.6</td>
<td>9.5±8.3</td>
<td>8.5±3.2</td>
<td>0.429</td>
</tr>
<tr>
<td>Peak ACh CBF, PU</td>
<td>79.0±45.2</td>
<td>41.6±29.3*</td>
<td>33.2±17.3*</td>
<td>0.001</td>
</tr>
<tr>
<td>Peak SNP CBF, PU</td>
<td>102.5±55.2</td>
<td>65.1±31.6*</td>
<td>65.1±31.9*</td>
<td>0.001</td>
</tr>
<tr>
<td>ACh CVC increase, %</td>
<td>698.7±382.1</td>
<td>402.4±239.9*</td>
<td>317.5±243.1*</td>
<td>0.001</td>
</tr>
<tr>
<td>SNP CVC increase, %</td>
<td>842.6±314.0</td>
<td>761.0±384.1*</td>
<td>560.5±270.7†</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are mean±SD. P value was estimated using a 1-way ANOVA followed by a post hoc Tukey multiple comparison test. *P<0.05 vs MetS-NT2D. †P<0.05 vs controls;

Discussion

Three major findings emerged from this study are as follows: (1) individuals diagnosed with MetS but without T2D exhibited depressed vascular function in both macro- and microvascular beds with serious implications for NO pathway. (2) The presence of T2D in MetS participants exacerbated the smooth muscle dysfunction in large and small vessels with no additional effect on endothelial function. (3) In association with abdominal obesity, inflammation and glucose intolerance were correlated with the vascular dysfunction, especially when the NO endothelium-dependent pathway was considered in the large brachial artery and skin microcirculation. These findings strengthen the evidence base for investigations into early generalized endothelial and vascular smooth muscle dysfunction in prediabetic MetS individuals to limit the risk of developing atherosclerosis disease.

Because endothelial dysfunction is a systemic disorder, peripheral vascular beds represent convenient access and require less elaborate equipment than assessments involving the vasoreactivity of coronary arteries. FMD has been widely used to assess vascular function and is a demonstrated predictor of CVD. However, vascular dysfunction may occur differentially in vascular beds. Specifically, endothelial cells differ in phenotype and structure depending on vessel type and have also produced heterogeneous responses to ex vivo isolated arteries investigation. Skin microcirculation has, therefore, been proposed as a model of systemic microvascular function to permit investigation of underlying mechanisms in CVD.

Systemic vascular disorders have been assessed by skin capillary density and pulse wave velocity in MetS, but the functional underlying mechanisms remained uncertain. Thus, to our knowledge, the current study focuses on an association between macro- and microvascular NO reactivity in MetS. It also demonstrates that not only endothelium NO–dependent but also endothelium NO–independent vasodilation impairments coexist, even without the presence of T2D. Our findings in the brachial artery (ie, FMD) are in accordance with the Framingham and other studies in MetS individuals, reporting endothelial-dependent dysfunction, independent of age and sex. Previous studies in T2D have postulated that microvascular dysfunction might cause an insufficient increase in shear stress to stimulate the endothelial NO release, demonstrated by lower FMD. Indeed, investigations into small vessels in the current study showed that CBF was decreased in response to acetylcholine in MetS with and without T2D when compared with controls. This suggested the presence of an impairment of NO-related endothelial function in the microvasculature in accordance with previous studies in skin and abdominal subcutaneous microcirculation. Moreover, a strong correlation was previously reported from an investigation into the endothelial function of the skin microcirculation in response to acetylcholine iontophoresis and the brachial arterial endothelial reactivity assessed by FMD, in a nonselected population. We observed a more modest correlation (r=0.51; P<0.001), which could be explained by our well-phenotyped MetS participants.

We postulate that both NO synthesis pathways are affected by MetS with or without the presence of T2D. Nevertheless, this endothelium-dependent dysfunction has to be interpreted with caution, given that the endothelium-dependent response includes, at least in part, the function of the smooth muscle.
Indeed, our results indicate that the smooth muscle response to exogenous organic nitrate is impaired in the brachial artery (NMD) and microvasculature (SNP) of patients with MetS. To minimize confusion in the results, we used an endothelium-dependent dilation index to isolate the part of vessel dilation attributed to the endothelium. When compared with healthy participants, we observed a decreased endothelium-dependent dilation index in patients with MetS showing an intrinsic endothelial-dependent dysfunction independently of smooth muscle cell vasodilation reserve, and without an exacerbated effect of T2D. In contrast to the current study, previous studies have been unable to describe smooth muscle dysfunction in MetS either during in vitro studies of the abdominal subcutaneous small vessels or in vivo in the skin microcirculation. These discrepancies could be explained by the inclusion of different tissues, or, a low number of participants in the first study. In the other 2 studies, the lack of insulin resistance or T2D among participants that was associated with the absence of cutaneous vascular dysfunction may have limited the capacity to describe smooth muscle dysfunction in MetS. When compared with the healthy controls, macrocirculation and microcirculation responses were, respectively, 24.1% and 32.2% lower in MetS-T2D and 13.5% and 8.0% in MetS-NT2D. These results are in accordance with our recent meta-analysis, showing that vascular smooth muscle function was more compromised in the microcirculation than in the macrocirculation of T2D individuals. Accordingly, the aggravation of VSM dysfunction observed in the microcirculation of individuals with T2D may be a distinctive feature of this metabolic disorder. However, this result has only previously been reported in animals. Thus, we also postulate that individuals with MetS-T2D are at higher risk of developing CVD than previously thought because vascular smooth muscle dysfunction has been proposed as a predictor of future cardiovascular events, with greater sensitivity than endothelial function.

Advancing the understanding of the pathophysiological link between macro- and microvascular dysfunction in individuals with MetS is complex because MetS is a cluster of symptoms. In this context, we also observed that mean arterial pressure is correlated with macrovascular endothelial function. Microvascular dysfunction is generally presented as a consequence of hypertension that could contribute to lower capillary density and arteriolar constriction, essentially mediated through higher endothelin-1 synthesis. However, recent evidence points to impaired microvasculature increasing peripheral resistance, possibly contributing to the development of high blood pressure in large vessels. In agreement with this hypothesis, we reported lower cutaneous vascular conductance response to acetylcholine and SNP iontophoresis in MetS participants, indicating higher skin microcirculation resistance, as well as a significant correlation between mean arterial pressure and FMD. Interestingly, we also observed that CBF in response to acetylcholine iontophoresis was an independent predictor of conduit artery endothelium-dependent vasodilation in patients with MetS. Thus, once installed, hypertension may lead to arteriolar wall remodeling and exacerbate microvascular function. Previous studies in patients with MetS without hypertension reported endothelium-dependent dysfunction in microcirculation. However, because the current study was only cross-sectional and most of our participants were hypertensive (73%), additional studies are needed.
to understand the complex interplay between microvascular dysfunction and chronic hypertension better.

Because microvessels and surrounding tissues are highly coupled, this functional study of microcirculation related to metabolic risk factors has generated much interest. However, the understanding of their association with vascular tree dysfunction in MetS remains incomplete. In the present study, central fat or active plasminogen activator inhibitor-1 proinflammatory cytokine emerged as independent predictors of FMD and CBF response to acetylcholine and SNP iontophoresis. Several hypotheses, which align with the present study’s findings, may help elucidate these mechanisms. Increasing evidence supports the role of abdominal adiposity in active endocrinologic functions via dysfunctional adipocytes by promoting a proinflammatory state leading to perturbed vascular homeostasis.28 Increased body mass index, central fat, adipokine levels, and decreased adiponectin were observed in our MetS participants, and significant relationships were shown between some markers of systemic inflammation, such as active plasminogen activator inhibitor-1, interleukin-6, high-sensitivity C-reactive protein, and tumor necrosis factor α and endothelium-dependent and endothelium-independent function in both macro- and microvascular beds. Elevated proinflammatory cytokines could explain the depressed vascular dysfunction in macro- and microcirculation beds. This may occur via the known deleterious effect of adipokines on many responses: (1) endothelial NO synthase expression in the endothelial cell resulting in lower NO synthesis and increased endothelin-1,30 (2) the anticontractive properties of healthy adipose tissue surrounding blood vessels,31 (3) higher proliferation and contractility of vascular smooth muscle cells,32 or (4) tetrahydrobiopterin bioavailability resulting in endothelial NO synthase uncoupling, leading to an increase in superoxide anion production and inhibition of endothelial NO synthase/cGMP signaling.33 Nonetheless, it must be considered that our study was performed in vivo during normal circulation in humans, and it was not possible to gain more direct insights into release of cytokines. Additional studies are needed to understand the cross talk between proinflammatory tissue and vascular function better.

| Parameters         | ACh CBF, % Value | P Value | FMD, % Value | ACh CBF, % Value | P Value | FMD, % Value | ACh CBF, % Value | P Value | FMD, % Value | ACh CBF, % Value | P Value | FMD, % Value | ACh CBF, % Value | P Value |
|--------------------|-----------------|---------|--------------|-----------------|---------|--------------|-----------------|---------|--------------|-----------------|---------|--------------|-----------------|---------|--------------|-----------------|---------|
| Central fat        | −0.42           | <0.0001 | −0.40        | 0.0001          | −0.37   | 0.0009       | −0.38           | 0.0003 |
| Triglycerides      | ...             | ...     | −0.29        | 0.005           | −0.28   | 0.0042       | ...             | ...     |
| HDL                | 0.25            | 0.010   | 0.23         | 0.023           | 0.29    | 0.002        | ...             | ...     |
| HbA1c              | −0.24           | 0.013   | −0.26        | 0.012           | −0.27   | 0.006        | ...             | ...     |
| PAI–1 active       | −0.30           | 0.002   | −0.30        | 0.004           | ...     | ...          | −0.34           | 0.001  |
| IL-6               | −0.25           | 0.010   | −0.39        | 0.0001          | −0.27   | 0.006        | ...             | ...     |
| hsCRP              | −0.24           | 0.014   | ...          | ...             | ...     | ...          | ...             | ...     |
| TNF-α              | −0.23           | 0.033   | 0.002        | ...             | ...     | ...          | ...             | ...     |
| Mean arterial      | ...             | ...     | −0.21        | 0.036           | ...     | ...          | ...             | ...     |

Ach indicates acetylcholine; CBF, cutaneous blood flow; FMD, flow-mediated dilation; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; IL-6, interleukin-6; NMD, nitrate-mediated dilation; PAI-1, plasminogen activator inhibitor-1; SNP, sodium nitroprusside; and TNF-α, tumor necrosis factor α.

Limitations
The present study has limitations. The cutaneous microcirculation may raise questions about its overall significance for small vessel assessment. However, there is a substantial literature indicating that cutaneous microvascular function mirrors generalized systemic microvascular function.29 Capillary density measures would also be interesting to measure because capillary rarefaction seems implicated with microvascular dysfunction. Further prospective studies are required to clarify this issue. Acquiring the levels of NO may have provided greater insight into the characteristics of our groups in relation to vascular function measures, but this parameter was not measured in this study. Vasoreactivity assessment includes SNP, FMD, and NMD that are NO dependent.34 However, NO inhibition reduces only 30% to 40% of the microvascular vasodilatory response induced by acetylcholine,35 suggesting that other factors may contribute to vasodilation in the resistance vessels. Another limiting factor was the use of existing or previous medical treatments of our participants, which could also have affected vascular function and circulating markers. However, there were no significant alterations to the results when medication and smoking history or status were accounted for (data not shown).

Conclusions
Our study provides new insights into the mechanisms that may contribute to the pathogenesis of vascular dysfunction in prediabetic MetS without a history of incompatible diseases, such as CVD. Overall, we demonstrated generalized vasoreactivity impairment in patients with MetS even without T2D, with an exacerbated dysfunction in vascular smooth muscle reactivity in patients with MetS and T2D. The presence of fat, especially central and systemic inflammation, seems to be implicated in the pathogenesis of vascular dysfunctions in MetS. Overall,
the clinical implications of investigating macro- and microcirculatory responses are of interest because our findings extrapolated to other cardiovascular functions and could explain the early recognition of increased risk of CVD in MetS.

Acknowledgment
Dr Walther contributed to study concept and design, to acquisition, analysis and interpretation of data, drafting of the article, and critical revision of the article for important intellectual content, and takes responsibility for the integrity of the work as a whole. We express our sincere thanks to the men and women who participated in the study and to Sandrine Gayraud for technical support. The help from Anne Camus in the management of participants was also greatly appreciated.

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Disclosures
None.

References
Metabolic syndrome (MetS) is a clustering of cardiovascular risk factors, including central obesity, impaired fasting glucose, dyslipidemia, and hypertension. Endothelial and vascular smooth muscle dysfunctions are considered early abnormalities in the development of atherosclerotic process in cardiometabolic diseases. Because studies to date have excluded patients with MetS without type 2 diabetes mellitus (T2D), whether associated micro- and macrovascular dysfunction is present in MetS individuals without T2D (MetS-NT2D) is uncertain. In this study, we demonstrate that MetS individuals without T2D exhibited already depressed vascular function in both macro- and microvascular beds with serious implications for NO pathway, in association especially with abdominal obesity and inflammation. We also reported that the presence of T2D in MetS participants exacerbated the smooth muscle dysfunction in large and small vessels. These important findings provide direction for investigations into early generalized endothelial and vascular smooth muscle dysfunction in prediabetic MetS individuals to limit the risk of developing atherosclerosis disease.
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MATERIAL AND METHODS

Subjects and screening
The RESOLVE study\(^1\) was a clinical trial designed to investigate the effect of a lifestyle intervention of exercise and nutrition on abdominal fat and cardiometabolic risks in MetS patients (American National Institutes of Health database n° NCT00917917). A specific subprotocol was designed to assess the vascular function in macrocirculation and microcirculation in MetS. A total of 150 individuals were examined. However, 32 participants were excluded due to incomplete data. Thus, we conducted cross-sectional analyses in 78 individuals with IDF-defined MetS, aged 50-70 years: 53 patients without T2D (MetS-NT2D), 25 MetS with T2D (MetS-T2D), and 40 age and gender-matched controls. All participants provided written informed consent. The study protocol was approved by the University Hospital Ethics Committee of Saint-Etienne, France.

Clinical and anthropometric assessment
All participants completed medical examinations. Blood biology and anthropometric data were used to confirm the eligibility of participants who needed to be classified as MetS as defined by the International Diabetes Federation\(^2\). These tests for descriptive characteristics were conducted according to a protocol previously described\(^1\). Systolic and diastolic blood pressures were obtained with a digital sphygmomanometer (Carescape V100, Dinamap, GE technology, USA). Height, weight and waist circumference were measured. Body mass index (BMI) was calculated as weight/height\(^2\). Body composition was assessed by dual energy X-ray absorptiometry (DXA, Hologic QDR Delphi series; Waltham, USA) to assess total lean and fat mass and central fat mass. All DXA measures were conducted by the same investigator.

Biochemical assessment
Fasting blood samples were collected to assess serum concentrations of triglycerides, high-density lipoprotein (HDL), low-density lipoproteins (LDL) and fasting glucose. Blood samples were stored at -80°C prior to subsequent ELISA-based analyses (Millipore, Billerica, MA, USA) for insulin, pro-inflammatory cytokines (TNF-\(\alpha\) and IL-6, hsCRP), the adipokines, leptin and adiponectin, and PAI-1 active. Insulin resistance was estimated by the calculation of the homeostasis model assessment-insulin resistance (HOMA-IR) index (fasting plasma glucose x fasting plasma insulin)/22.5\(^3\).

Vascular function
For both macrovascular and microvascular reactivity assessments, the participants were examined in a supine position, after a 20 minute resting period, in a room maintained at 22-24°C, after at least three hours of fasting and abstinence from caffeine and strenuous exercise for 24 hours. Patients were asked not to smoke at least 12 hours before the vascular measures.

Ultrasound Imaging
All vascular measurements were achieved using high-resolution vascular ultrasonography (MyLab30, Esaote SpA, Firenze, Italy), with a 10-MHz multi-frequency probe. B-mode images and Doppler signals were simultaneously recorded with ECG data, and off-line
analyses were performed using dedicated software (MyLab desk 9.0, Esaote, Florence, Italy). Arterial diameter was measured on B-mode images in the part of the artery running perpendicular to the ultrasound beam. The operator searched for the largest diameter, strong wall signals, and the longitudinal section of the artery in each image. Time-averaged mean velocity (cm.s\(^{-1}\)) was recorded, at the same level, by pulsed wave Doppler with a 45-60° insonation angle. Measurements were corrected for the insonation angle, and the pulsed Doppler sample volume was adjusted to cover the entire width of the vessel. The high-pass Doppler frequency filter was kept at the lower value ensuring rejection of arterial wall motion artifacts, with a cut-off value usually below 100 Hz. The same, well-trained operator (GW) performed all measurements. All data were calculated as the average of 5 consecutive measurements. Volume blood flow (BF, mL.min\(^{-1}\)) was then calculated as the product of artery cross-sectional area, derived from mean diameter, and by time-averaged mean velocity. Brachial flow-mediated dilation (FMD) assessment was performed thereafter according to the International Brachial Reactivity Task Force Guidelines\(^4\). Briefly, a pneumatic cuff was put on the right forearm proximal to the elbow. The ultrasound probe was placed approximately midway between the antecubital and axillary regions. The cuff was then inflated to 250 Hg mm during 5 minutes before sudden cuff deflation induced post-ischemic hyperemia. Fifteen minutes later, baseline measurements were repeated to measure nitrate-mediated dilation (NMD), before 0.4 mg of isosorbide dinitrate (Isocard, Schwarz Pharma, Monheim, Germany), an endothelium-independent vasodilator, given sublingually. This procedure has been described in detail elsewhere\(^5,6\). FMD and NMD (%) were expressed as the percentage change of diameter after post-ischemic hyperemia and after nitrate administration, respectively, relative to the baseline diameter. BF during peak post-ischemic hyperemia, measured by the average of 5 cardiac cycles of highest peak systolic velocity after cuff release, was expressed as the percent increase from baseline (PORH, %). Shear rate (s\(^{-1}\)) was calculated as 4 x mean systolic velocity / mean diameter, to estimate the shear stress induced by hyperemia\(^7\). To normalize diameter variations, Δshear rate was calculated from peak brachial shear rate minus resting brachial shear rate. Within-participant coefficients of variation were 1.8% for arterial diameters, 13.2% for time averaged mean velocity, 12.7% for BF and 9.2% for FMD\(^5,8\).

Cutaneous microvascular measurements
Arterial blood pressure was monitored during microcirculation exploration with a digital sphygmomanometer (Carecase V100, Dinamap, GE technology, USA). Microvascular endothelium-dependent and independent vasodilation was assessed using a Laser Doppler flowmetry (LDF) in combination with transdermal iontophoresis technique as previously described on the ventral face of the forearm skin\(^9\). Briefly, the LDF probe emits and detects light scattered in the tissue and is partially backscattered by moving blood cells, causing a change in frequency. According to the Doppler principle, the proportion of non-shifted to shifted light generates tissue perfusion, related to the number of moving cells and the velocity of these. Forearm cutaneous blood flow (CBF) was measured by a LDF probe (Periflux 5000, Perimed, Sweden) with a drug delivery chamber system with a wavelength of 780 nm. Laser Doppler measurements were recorded continuously at frequency of 32 Hz using an interfaced computer with acquisition software (Perisoft, Perimed, Järfälla, Sweden). Calibration was
performed using colloidal latex particles whose Brownian motion provided the standard value (Motility Liquid, Perimed, Järfälla, Sweden). Skin temperature was monitored throughout and maintained at 31°C by the same LDF heating probe. Prior to commencing procedures for measuring microvascular reactivity, the arm was immobilized with a vacuum cushion to ensure the participant was positioned as previously recommended. To investigate the endothelium-dependent and independent vasodilation, the site was iontophoresed of graduated doses of acetylcholine (ACh) and sodium nitroprusside (SNP), respectively. Iontophoresis is a non-invasive method allowing the local migration of vasoactive drugs, that are electrically charged molecules, across the skin by use of a small electric current. A current controller delivering device (Perilont, Perimed, Järfälla, Sweden) was connected to the LDF probe and to a conductive hydrogel electrode (reference) to provide direct current for iontophoresis. Subsequently, basal CBF was measured over a 5-minute period, iontophoresis of ACh or SNP was performed (200 µl of 2% solution) in order to achieve a plateau of the response. ACh was administrated with an anodal current of 0.1 mA with 7 successive doses of 20sec, with an interval of 60 seconds. SNP was administrated with a cathodal current with one dose of 0.02 mA during 20 minutes to provide effective SNP delivery; avoiding nonspecific vasodilation observed with higher cathodal electrical charges. CBF values were averaged over a 10 second period at the maximal plateau. Data were expressed as maximal percentage changes in perfusion versus basal values. Data were also expressed in maximal cutaneous vascular conductance (CVC), which is the flow in PU divided by the mean arterial pressure in mm.Hg to equalise differences in blood pressure between participants.

Data analyses
We calculated the percentage of endothelium-dependent dilatation (EDD index) related to maximal dilatation obtained with NO donors in micro- and macrocirculation to analyze the intrinsic relaxation due to endothelial cells. Following checks for normal distribution, data were presented as mean ± standard deviation. The distribution of categorical parameters was analysed by $\chi^2$. Arithmetic transformation was performed for non-Gaussian variables before parametric testing. Unpaired Student t-tests, analysis of variance (ANOVA) and covariance (ANCOVA), including gender as a covariate, were used to analyse controls vs. MetS participants. Tukey’s post-hoc test was used to assess inter-group differences in descriptive variables. Pearson’s $r$ correlation coefficient analyses were used to identify associations between metabolic risk factors, blood and vascular measures. Multiple stepwise linear regression analyses assessed the strongest predictors of variability in FMD, NMD, ACh and SNP. Significance was accepted at the $p<0.05$ level. Statistical procedures were performed by using MedCalc software (bvba, Mariakerke, Belgium).
REFERENCES


