Independent and additive effects of cytokine patterns and the metabolic syndrome on arterial aging in the SardiNIA Study

Angelo Scuteri a, *, Marco Orru b, Christopher Morrella a, Maria Grazia Piras b, Dennis Tauba a, David Schlessinger a, Manuela Uda b, Edward G. Lakatta a

a National Institute on Aging Intramural Research Program – NIH – Baltimore, USA
b Istituto di Neurogenetica e Neurofarmacologia (INN), Consiglio Nazionale delle Ricerche, c/o Cittadella Universitaria di Monserrato, Monserrato, Cagliari 09042, Italy

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A B S T R A C T

Objective: Metabolic syndrome (MetS) and its components accelerate age-associated increases in arterial stiffness and thickness. We investigated whether specific proinflammatory cytokines contribute to arterial aging, independent of age, sex, MetS, and other traditional CV risk factors.

Research design and methods: MetS components (ATP III criteria) and arterial properties were assessed in 6148 subjects, aged 14–102 in Sardinia, Italy. Common carotid artery (CCA) diameter, intima-media thickness (IMT), and aortic pulse wave velocity (PWV), adiponectin, leptin, high-sensitivity C reactive protein (hsCRP), monocyte chemoattractant protein 1 (MCP1), and interleukin 6 (IL6) were measured.

Results: While cytokine levels – except for MCP1 – were significantly higher (lower for adiponectin) in MetS than in control subjects, and the increased PWV and CCA IMT with aging were associated with MetS, this association was independent of cytokine levels (p < 0.001 for both PWV and CCA IMT). Specific cytokines, however, were significantly associated with arterial stiffness (higher leptin, p < 0.001, and higher hsCRP, p < 0.001) or thickness (lower adiponectin, p < 0.05, and higher IL6, p < 0.001) – independent of age, sex, MetS and other traditional CV risk factors. The co-occurrence of both MetS and higher cytokine levels was associated with greater increases in arterial stiffness and thickness.

Conclusion: While MetS and specific cytokine patterns associated with arterial aging, the increases in arterial stiffness and thickness are greater when both MetS and higher cytokine levels are present, suggesting a possible synergistic effect of MetS and inflammation on the arterial wall.

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1. Introduction

The metabolic syndrome (MetS) has been established as a significant risk factor for new onset diabetes mellitus and cardiovascular events [1], even in older subjects in whom MetS confers a 38% higher risk of myocardial infarction and stroke [2]. We have previously reported that the MetS is accompanied by accelerated central arterial aging [3,4], namely with increased arterial stiffness and thickness. Additionally, specific clusters of the components of the MetS are associated with a higher odds of having extremely stiff or thick arteries [4].

A growing body of literature suggests that diverse inflammatory and adipose tissue related cytokines correlated with arterial damage [5] and, thus, with endothelial dysfunction [6] and increased arterial stiffness and thickness [7–9]. Cytokine levels have been reported to be increased (decreased for adiponectin) in subjects with diabetes mellitus [10], and a proinflammatory status accompanies the presence of altered MetS components.

The aims of the present study were to determine: (a) whether MetS (and specific clusters of its components) and proinflammatory cytokines are associated with arterial stiffness and thickness independent of each other; after controlling for age, sex, traditional CV risk factors; (b) the simultaneous occurrence of both MetS and higher proinflammatory cytokine levels increase the likelihood for greater arterial stiffness or thickness.

2. Subjects and methods

2.1. Study population

The SardiNIA Study investigates the genetics of complex traits/phenotypes, including CV risk factors and arterial properties, in a Sardinian founder population [11,12]. Over a 3-year period, from November 2001 to December 2004, all residents aged 14 years and older in 4 towns of Sardinia Region, Italy were invited to participate in the study. The response rate was 60%, resulting in enrolment...
of 6148 participants aged 14–102 years old. Participants came to the clinic after fasting overnight, provided an informed consent, and after donating a blood sample, underwent a detailed medical history and full medical examination, including blood pressure and anthropometric measurements, a 12-lead resting EKG, measurements of arterial structure and function, and personality testing.

2.2. Variables measured

2.2.1. Blood pressure

Blood pressure determinations were performed in the morning, with subjects in the seated position, and following a 5 min quiet resting period. Blood pressure was measured in both arms with a mercury sphygmomanometer using an appropriately sized cuff. Values for systolic blood pressure (SBP) and diastolic blood pressure (DBP) were defined by Korotkoff phase I and phase V, respectively. The average of second and third measurements on both the right and left arm were used in the analysis. Pulse pressure was computed as PP = (SBP – DBP); mean BP was computed as MBP = DBP + (PP/3).

2.2.2. Anthropometry

Height, weight and waist circumference were determined for all participants. Body mass index (BMI) was calculated as body weight (kg)/height (m)^2.

2.2.3. Fasting plasma lipids and glucose

Blood samples were drawn from the antecubital vein between 7 and 8 AM after an overnight fast. Subjects were not allowed to smoke, engage in significant physical activity or take medications prior to the collection of the samples. Plasma triglycerides and total cholesterol, and fasting glucose were determined by commercially available kit [12]. LDL-cholesterol concentrations were estimated by the Friedewald formula.

2.2.4. Arterial structure and function

Aortic PWV was measured as previously described from simultaneous records of common carotid and femoral arteries flow waves [12]. The IMT was measured on the right common carotid artery (CCA) at end diastole by use of a linear-array 5–7.5-MHz transducer (HD1 3500- ATL Ultramark Inc.) as previously described [12]. Measurement was obtained from 5 contiguous sites at 1-mm intervals, and the average of the 5 measurements was used for analyses. All the measurements were performed by a single reader (AS). CCA systolic and diastolic diameter (d and D, respectively) were identified via ECG gating and measured similarly to IMT. CCA cross-sectional area (CSA) was calculated, as:

CCA CSA = \rho \times (\pi D^2 - \pi d^2)

where \rho is the arterial wall density (\rho = 1.06), Re = CCA IMT + CCA D [4].

2.3. Definition of the metabolic syndrome

The third report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III) [1] defined the MetS as an alteration in three or more of the following five components: abdominal obesity (W > 102 cm for men or > 88 cm for women), high triglycerides (T \geq 150 mg/dl), low HDL cholesterol (H < 40 mg/dl for men or < 50 mg/dl for women), elevated blood pressure (systolic or diastolic) (B \geq 130/\geq 85 mmHg), and elevated fasting glucose (G \geq 110 mg/dl).

2.4. Cytokine assays

Serum levels of hsCRP were measured by the high sensitivity Vermont assay (University of Vermont, Burlington), an enzyme-linked immunosorbent assay calibrated with WHO Reference Material. The lower detection limit of this assay is 0.007 mg/l, with an inter-assay coefficient of variation of 5.14%. Leptin (human serum adipokine – panel B; Lincoplex kit: Cat. # HADK2-61K-B) and adiponectin (human serum adipokine – panel A; Lincoplex kit: Cat. # HADK1-61K-A) were measured with a multiplex testing Luminex Model no. Luminex 200 IS Serial No. LX10006265401. Serum levels of IL-6 and MCP-1 were measured by Quantikine High Sensitive Human Immunoassays (R&D Systems, Inc.), according to the manufacturer’s instructions. This method employs solid-phase ELISA techniques. For IL-6, the lower detection limit is 0.039 pg/ml. The intra-assay coefficients of variation (CVs) were 6.9–7.8% over the range 0.43–5.53 pg/ml. For MCP-1, the lower detection limit is 5.0 pg/ml. The intra-assay coefficients of variation (CVs) were 4.7–7.8% over the range 76.7–1121 pg/ml.

2.5. Statistical analysis

Data are presented as mean ± SD unless otherwise specified. All analyses were performed using the SAS package for Windows (9.1 Version Cary, NC). Differences in mean values for each of the measured variables amongst groups were tested by ANOVA, followed by Bonferroni’s test for multiple comparisons. An ANCOVA was used to assess differences in mean values of arterial properties in subgroups of subjects stratified according to the presence of MetS and/or extreme values of individual cytokines, after controlling for age, sex, individual components of MetS, and LDL cholesterol. Univariate and multivariable linear regression analyses for continuous variables, or logistic regression for categorical variables, were conducted to detect associations of covariates and arterial structure or function. These models were constructed with arterial stiffness or thickness, alternatively, as dependent variables. The model fit for logistic regression was verified using the Hosmer and Lemeshow goodness of fit test. A two-sided p value < 0.05 indicated statistical significance.

3. Results

3.1. Cytokine levels in MetS and control subjects

Cytokine levels, except for MCP1, differed between control subjects and MetS (p < 0.0001), after controlling for age and sex. Specifically, adiponectin levels were lower, whereas leptin, hsCRP, and IL6 levels were dramatically higher in MetS than in control subjects (Table 1). Differences in MetS components and arterial wall characteristics are listed in Supplement Table 1. These differences are similar to those noted previously [4]. In univariate analysis, cytokines were significantly correlated with MetS and its components (Supplement Table 2). For some cytokines the correlation was stronger with lipids (adiponectin), or with elevated blood pressure (MCP1, IL6), or adiposity (leptin). Additionally, the prevalence of subjects in the upper quartile (lower quartile for adiponectin) for cytokines increased as the number of altered MetS components increased (Supplement Fig. 1). Any altered component of MetS was associated with a higher prevalence of extreme cytokine values.

3.2. Cytokine levels and arterial PWV and IMT

Univariate analyses showed that all cytokines were significantly and positively associated with PWV, PWV/MBP, CCA IMT, and CCA CSA (Supplement Table 3). These associations remained highly significant even after adjustment for age and sex. The association of
Multiple regression analyses to identify the role of MetS or proinflammatory cytokines as independent determinants of arterial function (PWV) and structure (CCA IMT).

Table 2
Comparison of cytokine levels in control subjects and in those with MetS.

<table>
<thead>
<tr>
<th>C</th>
<th>MetS</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5724</td>
<td>399</td>
</tr>
<tr>
<td>Age</td>
<td>43 ± 17</td>
<td>60 ± 13</td>
</tr>
<tr>
<td>Male sex</td>
<td>42.3</td>
<td>46.4</td>
</tr>
<tr>
<td>Adiponectin (mg/ml)</td>
<td>2.8 ± 1.9</td>
<td>2.4 ± 1.6</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>7465 ± 7354</td>
<td>11587 ± 10520</td>
</tr>
<tr>
<td>hsCRP (mg/ml)</td>
<td>2.5 ± 3.6</td>
<td>4.4 ± 5.2</td>
</tr>
<tr>
<td>MCP1 (pg/ml)</td>
<td>277 ± 168</td>
<td>303 ± 178</td>
</tr>
<tr>
<td>IL6 (pg/ml)</td>
<td>2.9 ± 2.5</td>
<td>4.4 ± 3.1</td>
</tr>
<tr>
<td>Adiponectin 25th percentile (&lt;1.54 mg/dl) (%)</td>
<td>25.9</td>
<td>35.3</td>
</tr>
<tr>
<td>Leptin 75th percentile (&gt;1.00 pg/ml) (%)</td>
<td>22.6</td>
<td>36.6</td>
</tr>
<tr>
<td>hsCRP 75th percentile (&gt;3.07 mg/ml) (%)</td>
<td>22.0</td>
<td>44.1</td>
</tr>
<tr>
<td>MCP1 75th percentile (&gt;331 pg/ml) (%)</td>
<td>23.9</td>
<td>30.6</td>
</tr>
<tr>
<td>IL6 75th percentile (&gt;3.64 pg/ml) (%)</td>
<td>22.5</td>
<td>45.1</td>
</tr>
<tr>
<td>No. of cytokine in the extreme quartile (%)</td>
<td>30.3</td>
<td>9.8</td>
</tr>
<tr>
<td>1</td>
<td>36.7</td>
<td>29.8</td>
</tr>
<tr>
<td>2</td>
<td>21.8</td>
<td>29.6</td>
</tr>
<tr>
<td>3</td>
<td>8.5</td>
<td>20.3</td>
</tr>
<tr>
<td>4-5</td>
<td>2.7</td>
<td>10.5</td>
</tr>
</tbody>
</table>

* After adjustment for age and sex.

| Table 1 Multiple regression analyses to identify the role of MetS or proinflammatory cytokines as independent determinants of arterial function (PWV) and structure (CCA IMT). |
|---------------------|---------------------|---------------------|---------------------|
|                      | PWV                  | CCA IMT              |                      |
|                      | Model 1              | Model 2              | Model 1              | Model 2              |
|                      | Beta ± SE            | p                   | Beta ± SE            | p                   |
| Age                  | 6.34 ± 0.16 $        | 6.20 ± 0.18 $       | 40.5 ± 0.9 $        | 39.5 ± 1.0 $         |
| Female sex           | −23.8 ± 4.8 $        | −13.5 ± 6 $         | 185.6 ± 25.9 $      | 208.9 ± 32.2 $       |
| Waist                | 2.12 ± 0.22 $        | 1.70 ± 0.25 $       | 2.2 ± 1.2 $         | 0.06 ± 0.18 $        |
| SBP                  | 2.95 ± 0.18 $        | 2.97 ± 0.18 $       | 8.5 ± 0.9 $         | 8.2 ± 1.0 $          |
| DBP                  | −1.01 ± 0.29 $       | −0.99 ± 0.31 $      | −9.6 ± 1.6 $        | −8.7 ± 1.6 $         |
| LDL chol             | 0.05 ± 0.06 $        | 0.07 ± 0.07 $       | −1.2 ± 0.3 $        | −0.8 ± 0.4 $         |
| HDL chol             | 0.10 ± 0.15 $        | 0.24 ± 0.16 $       | −2.8 ± 0.8 $        | −2.7 ± 0.9 $         |
| Triglycerides        | 0.05 ± 0.04 $        | 0.04 ± 0.05 $       | −0.5 ± 0.2 $        | −0.6 ± 0.2 $         |
| F glucose            | 0.49 ± 0.10 $        | 0.51 ± 0.11 $       | 0.3 ± 0.5 $         | 1.0 ± 0.6 $          |
| MetS                 | 51.1 ± 10.0 $        | 46.9 ± 10.5 $       | 224.6 ± 53.6 $      | 194.0 ± 56.2 $       |
| Adiponectin          | −1.31 ± 1.30 $       | −       | −       | 14.9 ± 6.6 $         |
| Leptin*              | −11.6 ± 3.3 $        | −       | −       | 30.7 ± 17.7 $        |
| hsCRP*               | −2.20 ± 0.63 $       | −       | −       | −0.9 ± 3.4 $         |
| MCP1*                | −0.02 ± 0.01 $       | −       | −       | 0.1 ± 0.1 $          |
| IL6*                 | 1.41 ± 0.96 $        | −       | 18.3 ± 5.1 $ |

* Multiplied $10^{-4}$, ** p < 0.05, *** p < 0.01, **** p < 0.0001.

*proinflammatory* cytokines and stiffer and thicker arteries was further investigated by comparing PWV and CCA IMT values in subjects with varying numbers of cytokines with extreme values. As shown in Supplement Table 4, after controlling for age, sex, and traditional CV risk factors, having 3 or more cytokines with extreme values was associated with significantly stiffer and thicker arteries. Out of all the combinations of ≥3 MetS components tested, some specific clusters of altered cytokines were preferentially associated with increased arterial thickness and stiffness (Supplement Table 4 right columns).

3.3. Concurrent vs. independent associations of MetS and cytokines and arterial stiffness and thickness

Are MetS and cytokine associations with accelerated arterial aging independent of each other?

Multiple regression models were constructed with arterial stiffness or thickness, alternatively, as dependent variables. The first set of models included age, sex, LDL cholesterol levels, and individual components of the MetS as covariates; in the second set of models, full cytokine panel was added as a covariate. In Model 1, age, sex, MetS ($p<0.0001$), together with waist circumference, SBP, DBP, and fasting glucose levels, were all significantly associated with PWV (Table 2, Model 1). Addition of the full panel of cytokine panel to the multivariable regression model (Model 2) did not change the impact of the above mentioned significant variables on PWV (Table 2, Model 2). Leptin ($p<0.001$) and hsCRP ($p<0.0001$) levels emerged as additional significant determinants of arterial stiffness, independent of MetS and its components. Similar results were observed when PWV was normalized for MBP, although the model ($R^2=0.38$) was lower than that observed for “absolute” PWV.

Age, sex, MetS ($p<0.0001$) together with waist circumference, SBP, DBP, HDL cholesterol, and triglyceride levels were all significantly associated with CCA IMT (Table 2, Model 1). Addition of the full panel of cytokine levels to the multivariable regression model (Model 2) attenuated the impact of waist circumference, HDL and LDL cholesterol levels on CCA IMT levels. Adiponectin ($p<0.05$) and IL6 ($p<0.0001$) levels emerged as significant determinants of CCA IMT, independent of MetS and its components (Table 2, Model 2). Similar results were observed when CCA IMT was expressed as CSA – thus account-
ing for arterial diameter, and the model $R^2$ (0.51) was higher than that observed for CCA IMT ($R^2 = 0.48$). When secondary analyses were run with further adjustment for smoking status, the results remained virtually unchanged for both PWV and CCA IMT.

Thus, multiple regression analysis models above confirmed that the association of MetS and arterial stiffness and thickness is independent of cytokine levels. Yet, specific inflammatory cytokines play and independently associated with these arterial properties.

To test for a possible interaction between cytokines and MetS to accelerate increasing arterial stiffness or thickness, we performed an ANCOVA analysis with age, sex, levels in LDL cholesterol and MetS components, and cytokine interaction terms between MetS and cytokine. The interaction terms between MetS and leptin or hsCRP (for PWV) and/or adiponectin or IL6 (for CCA IMT) were significant ($p < 0.001$). To better visualize the interaction between MetS and specific cytokines on arterial stiffness and thickness, average levels of PWV and CCA IMT in four subgroups defined by the presence/absence of MetS and by the presence/absence of individual cytokine levels in the upper quartile (the lower for adiponectin) are presented in Fig. 1. Higher leptin was associated with higher PWV in both subjects with or without MetS ($p < 0.001$) (Fig. 1 top left panel). A higher hsCRP level was associated with a higher PWV in control ($p < 0.001$) but not MetS subjects (Fig. 1 top right panel).

With regard to CCA IMT, low adiponectin was associated with higher CCA IMT only in subjects with MetS ($p < 0.001$) (Fig. 1 bottom left panel), whereas higher IL6 level was accompanied by higher CCA IMT in control, but not in MetS subjects (Fig. 1 bottom right panel). Thus, the co-occurrence of both MetS and specific higher cytokine levels was associated with greater increases in arterial stiffness and thickness.

### 3.4. Are specific clusters of MetS component having differing, cytokine levels differentially associated with arterial function and structure?

Supplement Tables 5–7 summarize the metabolic, arterial properties, and cytokine profile of subjects according to their clusters of MetS components.

Finally, we estimated the role of cytokines in the arterial burden associated with specific clusters of MetS components. The 2–2.5 fold higher odds of presenting an extremely high PWV that was associated with clusters of T-B-W or G-B-W or G-T-B (-W) components, as previously described [4], was not affected by cytokine levels. Similarly, the 2.5–3 fold higher odds of extremely thick CCA IMT associations with clusters of altered H-B-W or G-T-B (-W) components, as previously described [4], was also not affected by cytokine levels.

### 4. Discussion

A major strength of the present study is the large study population, its broad age range, and inclusion of key arterial parameters and of several cytokines in addition to MetS components. Indeed, most published studies investigating the relationship of cytokines with arterial properties have focused on fewer cytokines, introduced individually (i.e. not simultaneously) in the predictive models, often without including MetS components amongst the covariates.

The main findings of our study are that: [1] cytokine levels – except for MCP1 – differ between control subjects and MetS; [2] MetS remained significantly associated with arterial aging, independent of age, sex, and levels of individual MetS components; [3]
the association between MetS and arterial stiffness or thickness, however, is independent of cytokine levels; [4] nonetheless, specific cytokines are independently associated with arterial stiffness (higher leptin and higher hsCRP) or thickness (lower adiponectin and higher IL6) – independent of age, sex, and other traditional CV risk factors; [5] the presence of both MetS and elevated cytokine levels is associated with a much greater stiffening and thickening of central arteries.

Cytokine levels have been reported to be increased (decreased for adiponectin) in subjects with altered components of MetS or diabetes mellitus [10], and there is evidence to support their possible role as causative factors or mediators of vascular damage observed in MetS [5]. Cytokines are involved in complex signaling pathways and some of them regulate the elaboration of others, for example, IL6 stimulates hepatic production of hsCRP. The present study confirms that significantly lower level of adiponectin and higher levels of leptin, hsCRP, and IL6 occur in subjects with the MetS, as well as in different clusters of MetS components.

These findings further support the hypothesis that structural and functional changes in adipocytes may represent one of the primary alterations underlying the metabolic “derangement” leading to the metabolic syndrome and linking MetS with CV disease. Indeed, increased number, size, and turnover of adipocytes, infiltration of adipose by mononuclear cells, and rarefaction of blood vessels in the adipose tissue have been described as an early alteration in obesity [13]. Adipose tissue bioactive secreted products, for instance lower levels of adiponectin, have, in turn, been associated with reduced endothelium-dependent vasodilation [14] and higher risk of CV events [15,16].

With specific regards to arterial properties, prior studies had reported that CRP levels are associated with increased arterial stiffness in various study populations (normal subjects, hypercholesterolemia, hypertension, population study) [17–20]. Of note, in all of these studies – including the present one – CRP levels were within the normal range, reflecting an association of “subclinical inflammation” and arterial stiffness. In healthy adults, circulating levels of leptin are also associated with reduced arterial distensibility [21]. In some studies, adiponectin levels were inversely associated with CCA IMT [22–24]. The Malmo Study, for example, reported a gender-specific inverse relationship between circulating adiponectin levels and carotid IMT (significant in men, but not in women), but this became insignificant after accounting for MetS components [25]. In contrast, a lack of significant association between adiponectin and CCA IMT has been reported by the European group on insulin resistance [26]. These conflicting results may, at least in part, be explained by differences in selection of covariates or in the population-based study design.

The study design does not allow speculation about pathophysiological pathways linking selectively specific cytokines to arterial aging nor allow to hypothesize whether the increased levels of circulating inflammatory markers that we observed to be associated with arterial aging (adiponectin, leptin, hsCRP, IL6) may reflect inflammation generated by cells within the arterial wall [27], and not only cytokines derived from adipose tissue [28] – a key factor in the natural history of MetS [29].

4.1. Potential clinical impact

The present study shows that MetS and circulating cytokine levels are independently associated with accelerated arterial thickening and stiffening. Nonetheless, when MetS and elevated cytokine levels occur together, their impact on central arterial aging is additive resulting in much greater arterial stiffness and thickness. Thus, prevention or treatment strategies to reduce the risk of CV events associated with increased arterial stiffness and thickness might need to be more intensive in subjects presenting both sub-clinical inflammation, reflected in cytokine levels, and metabolic alterations that underlie MetS.

Conflict of interest

No author has any conflict to disclose.

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Contributions: AS contributed in conducting the analysis, researching data, and writing the manuscript. MO contributed in researching the data. CM contributed in reviewing/editing the manuscript. MGP and DT contributed in researching the data. DS and MU contributed in reviewing/editing the manuscript. EGL contributed to discussion, reviewing/editing the manuscript.

Appendix A. Supplementary data


References


