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## PAPER

# The central roles of obesity-associated dyslipidaemia, endothelial activation and cytokines in the Metabolic Syndrome — an analysis by structural equation modelling

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**HYPOTHESIS:** The multi-faceted components of the metabolic syndrome now include markers of inflammation and endothelial activation. Despite this growing body of epidemiological data, standard statistical methods fail to evaluate the nature of these associations adequately. In this pilot study, we hypothesize that obesity may lead to endothelial activation which is in part mediated by dyslipidaemia and proinflammatory cytokines. These factors interact to give rise to hyperinsulinaemia, hypertension and an anti-fibrinolytic state. To test this hypothesis, we used confirmatory factor analysis and structural equation modelling to fit these data to a model designed on theoretical grounds.

**METHODS:** Metabolic syndrome variables, cytokines (IL6 and TNF $\alpha$ ), markers of inflammation and endothelial activation were measured in 107 Caucasian non-diabetic subjects aged 40–75 y. Using confirmatory factor analysis, we identified six factors to represent composite measurements of blood pressure, obesity, dyslipidaemia, hyperinsulinaemia, endothelial activation and the anti-fibrinolytic state. We fitted these variables to two separate models, one using IL-6 and the other TNF $\alpha$  as the cytokine, and examined the inter-relationships (path analysis) amongst these variables, based on the above hypothesis.

**RESULTS:** Men were centrally more obese and had increased markers of endothelial activation, inflammation and the anti-fibrinolytic state as well as hyperinsulinaemia and dyslipidaemia, compared with women. Obesity indexes (both body mass index and waist–hip ratio) were strongly associated with multiple cardiovascular risk factors. Both IL6 and TNF $\alpha$  were correlated with age, male gender, obesity indexes and markers of endothelial activation. Only IL-6 was associated with smoking while TNF $\alpha$  was correlated with hyperinsulinaemia. In the TNF $\alpha$  model, 61% of the obesity variance was explained by male gender, 36% of TNF $\alpha$  variance by age and dyslipidaemia, 43% of dyslipidaemia variance by age and obesity, 33% of hyperinsulinaemia variance by dyslipidaemia and a non-smoking state, 29% of anti-fibrinolytic state variance by hyperinsulinaemia, 65% of endothelial activation variance by TNF $\alpha$ , dyslipidaemia and hyperinsulinaemia, 34% of blood pressure variance by hyperinsulinaemia and endothelial activation. In the IL-6 model, we observed similar relationships except that 23% of IL6 variance was explained by smoking and age.

**CONCLUSIONS:** Using confirmatory factor analysis and structural equation modelling, we found that obesity, dyslipidemia and cytokines were the principal explanatory variables for the various components of the metabolic syndrome, with IL6 and TNF $\alpha$  having different explanatory variables and effects. These complex inter-relationships were in part mediated by hyperinsulinaemia and endothelial activation. While this hypothetical model was based on scientific evidence, supported by rigorous analysis, it requires further confirmation in large-scale prospective studies. Given the complexity of the biological system and its interactions with exogenous factors, structural equation modelling provides a useful scientific tool for hypothesis testing, complementary to the more traditional experimental and cohort studies.

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**Keywords:** metabolic syndrome; interleukin-6 (IL6); tumor necrosis factor- $\alpha$  (TNF $\alpha$ ); hyperinsulinaemia; endothelial activation; obesity; dyslipidaemia; anti-fibrinolysis; structural equation modelling; factor analysis

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## Introduction

It has long been suggested that insulin resistance, hyperinsulinaemia and obesity play central roles in the metabolic syndrome characterized by clustering of multiple cardiovascular risk factors.<sup>1-7</sup> Other investigators have suggested that endothelial activation might antedate the clinical features of the metabolic syndrome.<sup>8-12</sup> Quantitative (high triglyceride and/or low HDL-C) and qualitative changes in lipoprotein particles (small dense and/or oxidized LDL-C) were associated with insulin resistance,<sup>8-12</sup> endothelial activation<sup>14,15</sup> and increased atherogenicity.<sup>16</sup> Subjects with the metabolic syndrome also have increased thrombotic and reduced fibrinolytic tendencies.<sup>17,18</sup> More recently, prospective cohort studies have demonstrated the predictive roles of inflammatory markers and cytokines including C-reactive protein and IL-6 on the subsequent development of glucose intolerance<sup>19,20</sup> and cardiovascular events.<sup>21,22</sup>

## Structural equation modelling

One of the major challenges in the study of this multifaceted syndrome relates to the complexity and collinearities of the relationships amongst these variables, which cannot be unravelled by standard statistical analysis. Structural equation modelling is a powerful statistical tool, which combines factor analysis and mathematical modelling to test hypotheses consisting of interacting variables and pathways with reference to substantive theory. This method is widely employed in psychological, social, educational and management fields where indicator variables such as attitudes, performances and personality traits, which cannot be readily measured, have to be derived from questionnaires.<sup>23,24</sup> The inter-dependent nature of these variables is very similar to the complexity of a biological system, characterized by interactions among endogenous and exogenous processes. Hence, structural equation modelling can be a useful research tool to complement the more conventional experimental or cohort studies in our attempt to understand biological phenomena and human diseases.

In a biological system, it is often the norm rather than exception that there are complex interactions between causes and effects. For instance, the effects of factor B may be explained by factor A directly as well as indirectly by factor D via the effect of factor C. Furthermore, factor B itself may be an explanatory variable for other factors. These complex inter-relationships cannot be fully explored by standard correlation or regression analysis. Structural equation modelling has the advantages of dissecting these relationships, assessing the total effects of variables on one another as well as providing directions for associations between variables and estimating the strengths of these relationships in an integrated model.

In brief, structural equation modelling involves 2 phases. Firstly, in the factor analytic model, closely correlated variables are grouped as factors with composite measurements. This endeavour aims to minimize the problem of multi-

collinearity and reduce the size of the data set to attain simplicity for the model. In the second phase, structural equation modelling is used to examine the causal relationships (paths) between these interacting variables. The pre-defined hypothesis, based on substantive theory, may need to be further refined based on the results of the data analysis and available evidence. This series of modelling aim to identify key causal relationships so as to best summarize the ostensibly complex associations among the variables. During these processes, novel or plausible relationships which have not yet reached statistical significance but are consistent with the original hypothesis may need to be included to improve the overall fitness of the final model.

We have used this statistical method to analyse a database of more than 1400 subjects and shown that most of the variance of the components of the metabolic syndrome could be explained by family history, age and obesity.<sup>25</sup> In this paper, based on a large number of experimental and clinical studies, we have further developed a hypothesis, incorporating markers of chronic inflammation and endothelial activation to unify these diverse clinical observations, to be tested by structural equation modelling.

## The old hypotheses — insulin resistance and visceral obesity syndromes

Until recently, insulin resistance, as exemplified by defective insulin-mediated glucose uptake by muscle, has been a favourite candidate as the linking factor for this multifaceted syndrome.<sup>1</sup> This hypothesis is based on the pluripotent effects of insulin on intermediary metabolism, sodium and water homeostasis and cellular growth.<sup>26</sup> However, the relationships between insulin resistance and blood pressure are often inconsistent.<sup>27-30</sup> Moreover, insulin treatment<sup>31</sup> and high insulin concentration in patients with insulinoma<sup>32</sup> do not appear to result in high blood pressure. These findings are some of the major arguments against the insulin resistance hypothesis.

Other workers have emphasized the important linking role of obesity, especially visceral obesity, in this syndrome.<sup>2,33,34</sup> Age-related decline in growth hormone or sex steroids, as well as stress-related hypercortisolaemia, can lead to the deposition of visceral fat.<sup>35,36</sup> These adipocytes are metabolically more active than subcutaneous adipocytes. The increased free fatty acids (FFA) production can lead to insulin resistance<sup>37,38</sup> and the various hormonal abnormalities underlying the visceral fat deposition may contribute to the associated metabolic, renal and vascular manifestations.<sup>5,25</sup>

## Alternative hypothesis — dyslipidaemia, endothelial activation and cytokines as linking factors

More recently, cross-sectional and prospective studies have demonstrated relationships between cardiovascular risks, markers of inflammatory responses and endothelial activa-

tion.<sup>39–42</sup> These associations cannot be easily explained by the insulin resistance or obesity hypotheses. Some researchers have proposed that chronic low grade inflammation, either due to infective agents such as *Helicobacter pylori*<sup>43</sup> or non-infective causes such as smoking,<sup>44</sup> may be associated with increased production of cytokines and reactive proteins, which can activate the endothelium and cause vascular damage. On the other hand, increased FFA formation can lead to insulin resistance through fuel competition<sup>45</sup> as well as increased formation of triglyceride-rich lipoprotein particles and small dense LDL particles.<sup>16</sup> These lipid particles enhance the expression of adhesion molecules and activate the endothelium.<sup>46</sup> In adipostatic regulation, cytokines such as TNF $\alpha$  are released by adipocytes to promote lipolysis and reduce appetite. These cytokines can induce insulin resistance directly<sup>47</sup> and enhance the expression of adhesion molecules and a procoagulant state.<sup>41</sup>

### Hypothesis

Based on this large body of literature, we hypothesize that smoking, chronic inflammation and obesity can induce the formation of proinflammatory cytokines. Together with obesity-associated dyslipidaemia, these factors interact to give rise to hyperinsulinaemia and endothelial activation. The latter two factors can then lead to the development of hypertension and a procoagulant state (Figure 1a).

### Methods

To test this hypothesis, we used structural equation modelling to re-examine a database consisting of 107 Caucasian non-diabetic subjects during a follow-up investigation of cardiovascular risk factors.<sup>39,48,49</sup> In the present analysis, additional parameters including inflammatory and endothelial markers were included. In summary, we originally investigated subjects aged 40–75 y randomly selected from the age–sex registry of a north London general practice and, 36 months later, restudied 125 of those with normal glucose tolerance. All subjects who had any physical illness or who were taking anti-inflammatory drugs were excluded from this analysis. In 107 of the recall subjects, sufficient serum and plasma was available to study a range of other variables. These subjects were similar to the total population in age, gender ratio and levels of risk factors under investigation. The details of the study methods for anthropometry, blood pressure, daytime and overnight urinary albumin excretion rates, cardiovascular disease history and Minnesota code classification of electrocardiograms have been described previously.<sup>39,48,49</sup>

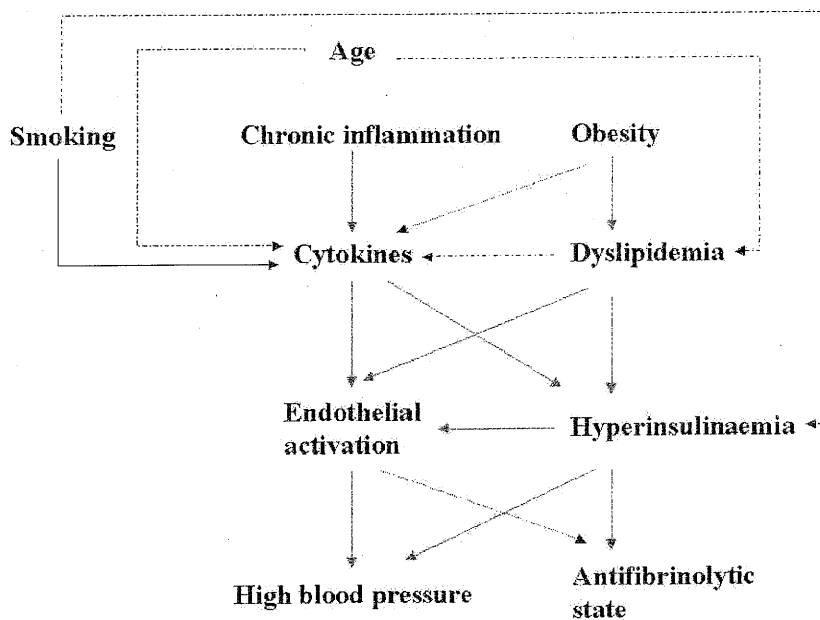
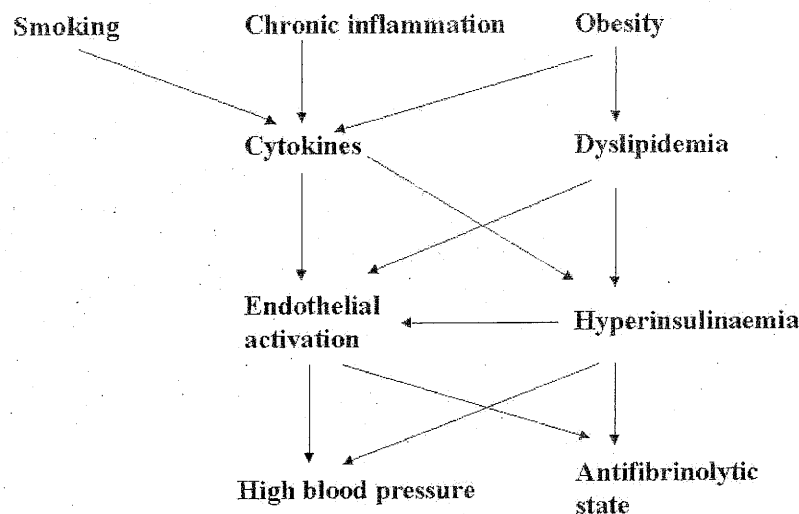
Fasting blood from these 107 subjects had been collected, spun at 2000g for 15 min before being separated, and frozen at  $-70^{\circ}\text{C}$ . The samples were assayed for total and high density lipoprotein cholesterol (HDL-C), triglycerides, insulin, proinsulin, des 31, 32 proinsulin and fibrinogen<sup>39,48,49</sup> as well as for additional measures of endothelial

and acute phase markers and other related variables. A separate blood sample was collected in iced citrate and then centrifuged at 2500g for 30 min at  $4^{\circ}\text{C}$  to obtain platelet poor plasma for the measurement of plasminogen activator inhibitor 1 (PAI-1). Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) were measured by enzyme-linked immunosorbent assays (ELISA; R and D Systems Ltd, Oxford, UK). Soluble vascular cell adhesion molecule-1 (VCAM-1), interstitial cell adhesion molecule-1 (ICAM-1), E-selectin, thrombomodulin (TM), von Willebrand factor (vWF), cellular fibronectin (CFN), tissue plasminogen activator (tPA) antigen, PAI-1 activity, PAI-1 antigen and C-reactive protein (CRP) were measured at the Gaubius Laboratory, TNO-PG, Leiden, The Netherlands. Soluble VCAM-1 and E-selectin were measured by commercially available kits obtained from Amersham International plc (Little Chalfont, UK). Soluble ICAM-1 was measured by commercial kit obtained from Bender MedSystems (Vienna, Austria). Thrombomodulin was assayed using an ELISA kit (Stago), vWF antigen by an ELISA method using polyclonal antibodies from DAKO (Glostrup, Denmark). Cellular fibronectin was measured by a sandwich ELISA using a monoclonal antibody IST-9 (Sera-Lab, Crawley Down, UK) against the ED-A domain for capture, and a peroxidase-conjugated polyclonal fibronectin antibody (DAKO, Glostrup, Denmark) for detection. Tissue plasminogen activator (tPA) and PAI-1 antigens were measured by ELISA (Organon Teknika, Turnhout, Belgium), which recognizes both free forms of the factors and complexes of tPA with PAI-1. C reactive protein was measured using a highly sensitive ELISA procedure with a range of 0.25–10.25  $\mu\text{g}/\text{ml}$  and an inter-assay coefficient of variation (CV) of 8%. Antibody titres to *Helicobacter pylori* were measured using an enzyme immunoassay (Helico-G, Porton Cambridge, Newmarket, UK). The coefficients of variations of these laboratory assays have been reported in detail previously.<sup>48</sup>

### Statistical methods and structural equation modelling

The statistical analysis was performed using the Statistical Package for Social Sciences (version 7) on an IBM-compatible computer. Plasma triglyceride, des 31, 32 proinsulin, proinsulin, urinary albumin, CRP, CFN, vWF, PAI-1, IL6, ICAM, VCAM, fibrinogen and *Helicobacter* antibody titre were logarithmically transformed due to skewed distributions. All data are expressed as mean  $\pm$  s.d. or geometric mean  $\times/\div$  antilog s.d. as appropriate. Comparisons of groups were performed using unpaired Student's *t*-test. Spearman's correlation analysis was performed to show the inter-relationships between variables.

Structural equation modelling via LISREL<sup>24</sup> is an appropriate technique for testing the hypothesized causal model (Figure 1a) and estimating parameters. In the confirmatory factor analytical model, the convergent validity of the factors is shown by a loading lambda value of the composite



**Figure 1** (a) A unifying hypothesis to explain the multi-faceted nature of the metabolic syndrome. Smoking, chronic inflammation and obesity associated dyslipidaemia lead to increased cytokine production, endothelial activation and hyperinsulinaemia. The latter three factors interact: and contribute to the development of high blood pressure and an antifibrinolytic state. (b) The final model which was derived after further refinement of the predetermined hypothesis, based on past research, theory and exploratory data analysis. The solid arrows represent paths that have been confirmed, the dotted arrows represent relationships that were not proven and the dashed line represent new paths that were identified during the modelling.

measurement greater than 0.3. The overall reliability of the factor is indicated by an alpha coefficient  $> 0.7$ . These factors are then subjected to structural equation modelling and fitted according to the hypothesis. The final model should contain minimal redundant and deviant findings but mostly significant, albeit not exclusively, factor loadings and path coefficients in a predicted manner consistent with theory and evidence. The model estimates and tests the

significance of total effects, which are the sums of direct and indirect effects through all mediating variables. The final model also gives the total variance of each factor as explained by other factors, which may interact with one another. The strength of relationships between two factors is indicated by the path ( $\beta$ ) coefficients with 95% confidence intervals. A satisfactory model usually has a high goodness of fit index (GFI) preferably higher than 0.9, a low likeli-

hood ratio chi square ( $L^2$ ) and root mean square residual (RMSR)  $< 0.05$ . A  $P$ -value less than 0.05 is considered to be significant.

## Results

Table 1 shows the clinical and biochemical variables, markers of inflammatory responses and endothelial activation in men and women. Men had a more adverse cardiovascular risk profile than women. These include central obesity, elevated concentrations of insulin-like molecules, cytokines, tPA, PAI-1 antigen, CFN, E-selectin, low HDL-C and small size of low density lipoprotein (LDL) particles. Compared with non-smokers, smokers had lower levels of fasting insulin ( $39.6 \pm 30$  vs  $25.6 \pm 12.9$  pmol/l,  $P < 0.001$ ), proinsulin ( $4.04 \pm 2.5$  vs  $3.03 \pm 1.52$  pmol/l,  $P < 0.05$ ) and des 31, 32 proinsulin ( $1.86 \pm 3.11$  vs  $1.07 \pm 2.5$  pmol/l,  $P < 0.05$ ) levels.

Tables 2 and 3 summarize the significant correlation coefficients ( $P < 0.05$ ) amongst these variables using simple correlation analysis. Table 1 shows the multiple correlations amongst conventional cardiovascular risk factors such as obesity, insulin, blood pressure and lipid parameters with body mass index (BMI) and weight-to-hip ratio (WHR) as particularly strong correlates. Male sex, age and obesity indices were closely associated with inflammatory markers and cytokines. Smoking was associated with low insulin levels but increased IL6 and VCAM levels. Table 3 shows the inter-relationships between conventional risk factors, cytokines, markers of inflammation and endothelial activation. There were particularly strong correlations amongst CRP, TNF $\alpha$ , IL-6 and tPA with various cardiovascular risk factors, while TNF $\alpha$  was correlated with obesity indexes and insulin products, IL6 was associated with smoking. *Helicobacter pylori* titre did not correlate with any of the parameters.

In the confirmatory factor analysis, all lambda values of the composite measurements were greater than 0.3 and the  $\alpha$  coefficients of the factor were greater than 0.7, supporting the validity of these factors and their composite measurements. We were, however, unable to group IL6 and TNF $\alpha$  in the same factor of cytokines. Hence, two separate models using IL6 and TNF $\alpha$ , respectively, were tested. In both models, BMI, WHR and central distribution of subcutaneous fat were loaded with obesity; fasting triglyceride, low HDL-C and LDL-particle size, with dyslipidaemia; systolic and diastolic blood pressure with blood pressure; plasma glucose, insulin, proinsulin, des 31, 32 insulin with insulin parameters; CFN, vWF, TM and tPA with endothelial dysfunction; PAI-1 activity, PAI-1 antigen and tPA with anti-fibrinolytic state. Age, gender, smoking and *Helicobacter pylori* titre did not load with other measurements to identify new factors and were treated independently. Other variables including fibrinogen, CRP, factor VII, urinary albumin excretion, ICAM, VCAM, E-selectin and ECG findings were excluded from the factor analytical model.

Figures 2 and 3 show the two causal models using TNF $\alpha$  and IL6 as the cytokine parameters, respectively. These

models had GFI indexes of 0.75 and 0.76, respectively, and a RMSR of 0.121 and 0.119, respectively. These figures suggest a reasonable, though not perfect, fit of the data to these models. The explained variance of each variable and the path coefficients ( $\beta$ ) with 95% confidence intervals of the relationships amongst these variables are shown in the figures and legend. Both models explained significant amounts of variance of the major components of the metabolic syndrome. In the TNF $\alpha$  model, 61% of the variance in obesity was explained by male gender, 36% of TNF $\alpha$  by obesity, dyslipidaemia and age, 43% of dyslipidaemia by age and obesity, 33% of insulin parameters by dyslipidaemia, a non-smoking state and TNF $\alpha$ , 29% of the anti-fibrinolytic state by insulin parameters, 65% of endothelial activation by TNF $\alpha$ , dyslipidaemia and insulin parameters, 34% of blood pressure by insulin parameters and endothelial activation.

In the IL6 model, 60% of the variance in obesity was explained by male gender, 52% of dyslipidaemia by age and obesity, 23% of IL6 by smoking, age and dyslipidaemia, 35% of insulin parameters by dyslipidaemia and a non-smoking state, 26% of anti-fibrinolytic state by insulin parameters, 38% of endothelial activation by IL6, insulin parameters and dyslipidaemia, 31% of blood pressure by insulin parameters and endothelial activation.

## Discussion

### Univariate and correlation analysis

In this initial part of the analysis, as reported in most studies,<sup>50</sup> we observed correlations amongst conventional cardiovascular risk factors. Furthermore, obesity, male gender, ageing and smoking were associated with increased markers for endothelial activation and inflammation. In agreement with other researchers,<sup>19,48,51</sup> there were also associations between CRP, cardiovascular risk factors, cytokines and markers of endothelial activation. Nonetheless, we found that, while TNF $\alpha$  was associated with insulin parameters and obesity indexes, IL6 was more associated with smoking. These findings are in support of the linking role of TNF $\alpha$  in obesity-associated insulin resistance<sup>52</sup> and IL-6 as the main cytokine in low-grade inflammatory conditions.<sup>53,54</sup>

### Confirmatory factor analysis

It is important to point out that these correlations only generate hypotheses which require confirmation. Furthermore, due to the multicollinearity of these associations, factor analysis needs to be performed to reduce the size of the data set to improve the simplicity of the model. In the confirmatory factor analysis, six factors were identified which were reliable with high loadings of the composite measurements and high  $\alpha$  reliability coefficients of the factors. The factors consisting of composite measurements of blood pressure, lipid and insulin parameters and the anti-fibrinolytic markers were expected. The more dominant

effects of WHR over BMI were in accord with the known adverse effects of central adiposity on cardiovascular risks.<sup>55</sup> The inclusion of PAI-1 activity in both factors of the anti-fibrinolytic state and endothelial activation was consistent with the anti-fibrinolytic effects of PAI-1<sup>56</sup> and its expression by an activated endothelium.<sup>41</sup> In agreement with their different associations in the correlation analysis, we were unable to load IL6 and TNF $\alpha$  in the same cytokine factor. As a result, they were put into two separate models in our subsequent structural equation modelling.

Some variables including urinary albumin excretion, fibrinogen, factor VII and CRP did not cluster to identify factors, despite their known predictive values in cardiovascular risks.<sup>19,57,58</sup> Also, ICAM, VCAM and E-selectin, which are often used as markers of endothelial activation, did not reflect the factor of endothelial activation which included only TM, tPA, CFN and vWF. These findings may be in part due to the small sample size of these relatively healthy subjects with insufficient data variance. Other possible reasons include the uncertain validity of the measurements in

**Table 1** The clinical characteristics and markers of inflammation and endothelial activation in 107 Caucasian non-diabetic subjects

	Total (n = 107)		Male (n = 59)		Female (n = 48)	
	Mean	s.d.	Mean	s.d.	Mean	s.d.
<b>Demographics</b>						
Age (y)	59.0	10.9	60.4	11.4	57.2	10.0
Non-smokers (%)	65.4		66.1		64.6	
Current and ex-smokers (%)	34.6		33.9		35.4	
Systolic blood pressure (mmHg)	125	18	128	82	121	20
Diastolic blood pressure (mmHg)	80	11	82	11	78	10
Subjects with angina or ischemic changes on ECG (%)	14.0		10.2		18.8	
<b>Obesity indexes</b>						
Body mass index (kg/m <sup>2</sup> )	25.6	4.5	26.2	4.7	25.5	4.3
Waist-hip ratio	0.86	0.08	0.92	0.06	0.80	0.05 <sup>†</sup>
Subscapular-triceps skinfold ratio	1.31	0.59	1.69	0.50	0.84	0.22 <sup>†</sup>
<b>Lipid parameters</b>						
Plasma triglyceride (mmol/l) <sup>a</sup>	1.47	1.43	1.55	1.43	1.37	1.43
HDL-cholesterol (mmol/l)	1.38	0.37	1.25	0.37	1.54	0.31 <sup>†</sup>
LDL particle size (ÅU) (n=40)	266	7.2	264	7.3	271	4.9**
<b>Insulin parameters</b>						
Fasting plasma glucose (mmol/l)	4.48	0.47	4.53	0.53	4.42	0.38
Fasting plasma insulin (pmol/l)	34.8	26.2	42.7	29.9	24.7	15.9 <sup>†</sup>
Proinsulin (pmol/l) <sup>a</sup>	3.69	2.25	4.40	2.64	2.81	1.18 <sup>†</sup>
Des 31,32 proinsulin (pmol/l) <sup>a</sup>	1.89	2.02	2.42	2.16	1.37	1.76 <sup>†</sup>
<b>Albuminuria</b>						
Urine albumin (mg/l) <sup>a</sup>	2.00	1.28	2.07	1.32	1.94	1.22
<b>Acute phase proteins</b>						
Fibrinogen (mg/dl)	289	76	292	81	286	69
C reactive protein ( $\mu$ g/ml) <sup>a</sup>	1.46	1.83	1.47	1.68	1.44	2.02
<b>Markers of endothelial activation</b>						
Cellular fibronectin (%) <sup>a</sup>	106	1.90	119	1.63	92.7	2.18*
von Willebrand factor (%) <sup>a</sup>	103	1.45	104	1.44	101	1.46
Thrombomodulin (ng/ml)	35.9	17.4	38.2	17.3	33.5	17.5
Tissue plasminogen activator (ng/ml)	21.0	9.4	23.7	8.6	17.8	9.4 <sup>†</sup>
Interstitial cell adhesion molecule-1 (ICAM-1) (ng/ml) <sup>a</sup>	615	1.68	609	1.66	619	1.70
Vascular cell adhesion molecule-1 (VCAM-1) (ng/ml) <sup>a</sup>	277	1.40	288	1.42	263	1.37
E-selectin (ng/ml)	15.9	4.6	17.5	4.8	14.1	3.5 <sup>†</sup>
<b>Anti-fibrinolytic markers</b>						
Plasminogen activator inhibitor-1 activity (ÅU/ml) <sup>a</sup>	7.93	2.24	8.68	2.27	7.01	2.20
Plasminogen activator inhibitor-1 antigen (ng/ml) <sup>a</sup>	79.3	2.03	96.6	1.88	60.4	2.07 <sup>†</sup>
<b>Cytokines</b>						
Tumour necrosis factor- $\alpha$ (pg/ml)	3.88	1.32	4.25	1.42	3.41	1.03 <sup>†</sup>
Interleukin-6 (pg/ml) <sup>a</sup>	2.71	1.94	3.31	1.87	2.10	1.94**
<b>Infection markers</b>						
<i>Helicobacter pylori</i> titre (1/n) <sup>a</sup>	24.5	3.0	26.7	2.8	22.6	3.3

Mean  $\pm$  s.d. or <sup>a</sup>geometric mean  $\times / \div$  antilog s.d. \* $P < 0.05$ , \*\* $P < 0.01$ , <sup>†</sup> $P < 0.001$ .



**Table 3** Correlation matrix showing the significant correlation coefficients ( $P < 0.05$ ) amongst clinical characteristics and markers of inflammation and endothelial activation in 107 Caucasian non-diabetic subjects

	Fib	FVII	CRP	CFN	VWF	TM	TPA	ICAM	VCAM	E-sel	PAI-1	PAI-1 Ag	TNF $\alpha$	IL6
Age	0.237		0.300	0.338	0.336	0.243	0.336	0.425	0.215				0.465	0.302
Sex (male = 1; female = 2)		0.212		-0.192			-0.309					-0.336	-0.346	-0.283
Smoking									0.338					0.275
Systolic BP	0.306		0.345	0.284	0.284		0.389		0.288		0.203	0.231	0.332	0.308
Diastolic BP	0.265		0.270	0.197	0.197		0.287			0.266	0.330	0.354	0.268	
BMI	0.220		0.364				0.353				0.299	0.493	0.288	0.191
WHR			0.321	0.287			0.512		0.237	0.390	0.307	0.557	0.508	0.410
Skinfold ratio			0.209	0.270		0.207	0.403		0.203	0.310	0.216	0.377	0.368	0.259
Triglyceride (TG)							0.195				0.263	0.330	0.304	
HDL-cholesterol														
LDL size	0.355									-0.359		-0.513	-0.451	
Fasting glucose							0.204							
Fasting insulin							0.329				0.396	0.483	0.303	
Proinsulin														
Split insulin							0.321				0.331	0.438	0.208	
Albuminuria							0.230						0.246	0.201
Fibrinogen (Fib)	1.000		0.217											
Factor VII (FVII)		1.000												
C-reactive protein (CRP)	0.217		1.000	0.255	0.307		0.398		0.351			0.191	0.446	0.370
Cellular fibronectin (CFN)		0.255	1.000	0.284	0.284		0.371						0.361	
Von Willebrand factor (vWF)			0.307	0.284	1.000	0.232	0.276	0.412					0.370	
Thrombomodulin (TM)				0.232	1.000	1.000	0.250	0.318					0.297	
Tissue plasminogen activator (TPA)			0.398	0.371	0.276	0.250	1.000		0.232	0.266	0.403	0.481	0.404	0.315
ICAM					0.412	0.318		1.000					0.398	
VCAM							0.232		1.000	0.407			0.414	0.518
E-selectin (E-sel)							0.266		0.407	1.000			0.263	0.370
Plasminogen activator inhibitor-1(PAI-1) activity							0.403				1.000	0.220	0.207	
PAI-1 antigen (PAI-1 Ag)			0.191				0.481			0.220	0.680	1.000	0.348	
Tumor necrosis factor $\alpha$ (TNF $\alpha$ )			0.446	0.361	0.370	297	0.404	0.398	0.414	0.263	0.207	0.348	1.000	0.453
Interleukin-6 (IL-6)			0.370				0.315	0.518	0.518	0.370			0.453	1.000

reflecting true biological activities (such as short biological half-life, day-to-day or within-day variations, paracrine or intracellular effects), marked intra-individual variations of these markers as well as variations in laboratory assays and low sensitivity or specificity of these measurements.

### Structural equation modelling

**Gender and body composition.** Following the confirmatory factor analysis, we used structural equation modelling to examine the inter-relationships amongst these factors based on the hypothesis (Figure 1a). In both models (Figures 2 and 3), we demonstrated the important effect of male gender on obesity which was largely due to central adiposity. We were unable to examine this relationship in women and men separately due to small sample sizes. We were also unable to demonstrate the effects of age on obesity, which might have been overwhelmed by the effects of male gender on central adiposity. In this respect, there is evidence showing that age-related decline in free testosterone and increased sex hormone binding globulin are associated with increased visceral adiposity<sup>59</sup> and that treatment with androgen improved body fat distribution in ageing men.<sup>60</sup>

**Dyslipidaemia, endothelial activation and hyperinsulinaemia.** In support of the evidence that obesity induces insulin resistance through fuel competition and triglyceride deposition in muscle,<sup>61,62</sup> we detected the mediating effects of obesity on hyperinsulinaemia by dyslipidaemia in both models. Since insulin also inhibits the hepatic secretion of triglyceride-rich lipoprotein particles such as VLDL-C and promotes catabolism of VLDL-C to HDL-C,<sup>50</sup> a vicious cycle of insulin resistance and dyslipidaemia can therefore be set up. This form of triglyceride-rich dyslipidaemia is associated with increased formation of small dense LDL particles<sup>63</sup> which are more readily taken up by macrophages to form foam cells and atheromatous plaque.<sup>16</sup> These lipid particles are also more prone to oxidation to release chemotactic and prothrombotic factors such as ICAM, VCAM, CFN, vWF, TM and tPA from the endothelium as well as impair endothelial-dependent dilatation.<sup>64-67</sup> Besides, there is evidence showing that adipocytes can secrete a large number of vasoactive peptides including IL6, TNF $\alpha$ , CRP, angiotensin II and PAI-1 antigen,<sup>68</sup> which may contribute to endothelial activation and a proinflammatory state. Against this background, our finding that obesity-associated dyslipidaemia was one of the main explanatory factors for hyperinsulinaemia and endothelial activation lends further support to these increasingly popular notions.

**Cytokines, endothelial activation, hyperinsulinaemia.** In the final model, we were able to demonstrate a *direct but weak relationship* between TNF $\alpha$  and insulin parameters but not between TNF $\alpha$  and obesity. Given the autocrine or paracrine actions of TNF $\alpha$  in adipocytes, these circulating levels therefore may not fully reflect their biological effects. Besides, the

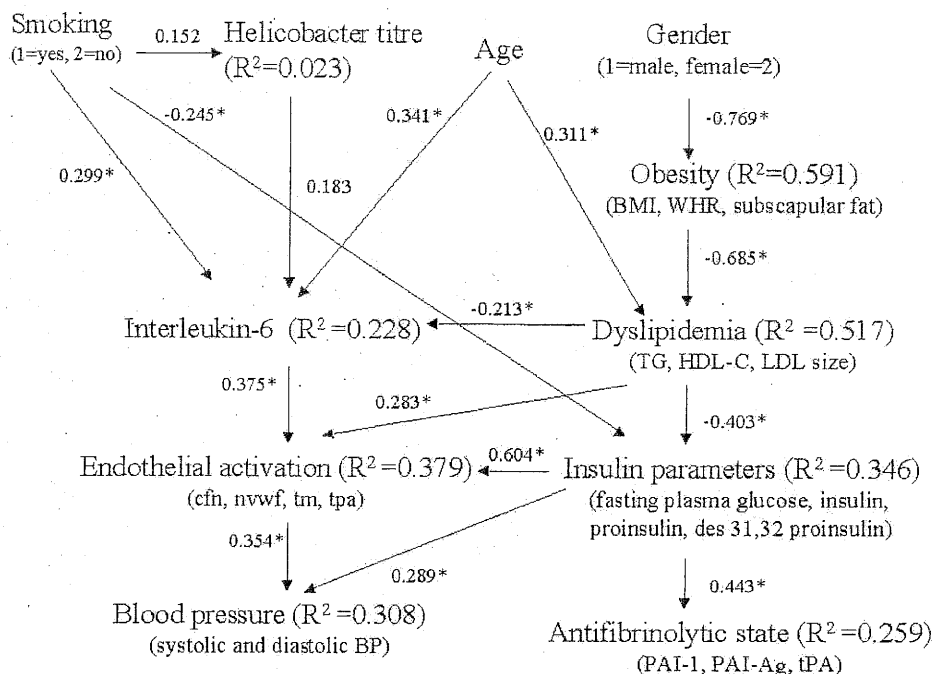
relationship between TNF $\alpha$  and obesity might have been confounded by that between TNF $\alpha$  and hyperinsulinaemia. In this regard, TNF $\alpha$  has been shown to cause insulin resistance by directly inhibiting glucose translocation and insulin signalling.<sup>69,70</sup>

On the other hand, we were able to show that smoking was an explanatory factor for IL6, but not TNF $\alpha$ . While these findings are compatible with the known effects of smoking on cytokines and endothelial activation,<sup>44,71</sup> it also highlights the potential differences in the determinants and effects of different cytokines in a biological system.

Although some workers have demonstrated the association between chronic infections and increased cardiovascular risks, in part mediated by cytokines,<sup>72,73</sup> this relationship has not been borne out in a large scale prospective study involving apparently healthy postmenopausal women.<sup>43</sup> In this relatively healthy population, we were unable to demonstrate associations between *Helicobacter pylori* titres, inflammatory markers or cardiovascular risk factors on simple correlation analysis. However, we found a weak relationship between *Helicobacter pylori* titres and cytokines in both models. In line with current evidence,<sup>54,65-67,74,75</sup> we also confirmed that both IL6 and TNF $\alpha$  were major explanatory variables of endothelial activation, thereby providing a link between low-grade infection and increased cardiovascular risks.

**Endothelial activation, hyperinsulinaemia, blood pressure and procoagulant state.** As predicted in our hypothesis, hyperinsulinaemia and endothelial activation were the main explanatory variables for blood pressure. Insulin has potential blood pressure elevating effects by activating the sympathetic nervous system,<sup>76</sup> promoting sodium and water retention<sup>77</sup> and stimulating vascular smooth muscle cellular growth.<sup>78</sup> All these effects can become clinically important in subjects who have reduced capacity to buffer these vasoactive effects.<sup>28</sup> In this respect, it has been suggested that abnormalities in the microvasculature with impaired endothelial-dependent vasodilatation may contribute to the development of vascular resistance in the pathogenesis of high blood pressure.<sup>79-81</sup>

In both models, hyperinsulinaemia was also the main explanatory variable of the anti-fibrinolytic state. Increased production of coagulation factors and inhibitors of the fibrinolytic pathways have been associated with hyperinsulinaemia.<sup>82,83</sup> Possible underlying mechanisms include the effects of VLDL-triglyceride on PAI-1 synthesis of the endothelial cells and hepatocytes, effects of increased insulin and proinsulin levels on PAI-1 synthesis and increased production of PAI-1 in the adipocytes of visceral fat.<sup>17</sup> A recent prospective study has also demonstrated that the prognostic role of PAI-1 activity in predicting coronary events was principally related to insulin resistance while that of tPA could be explained by other mechanisms including insulin resistance, inflammation and endothelial damage.<sup>18</sup> Although we were unable to confirm a direct relationship



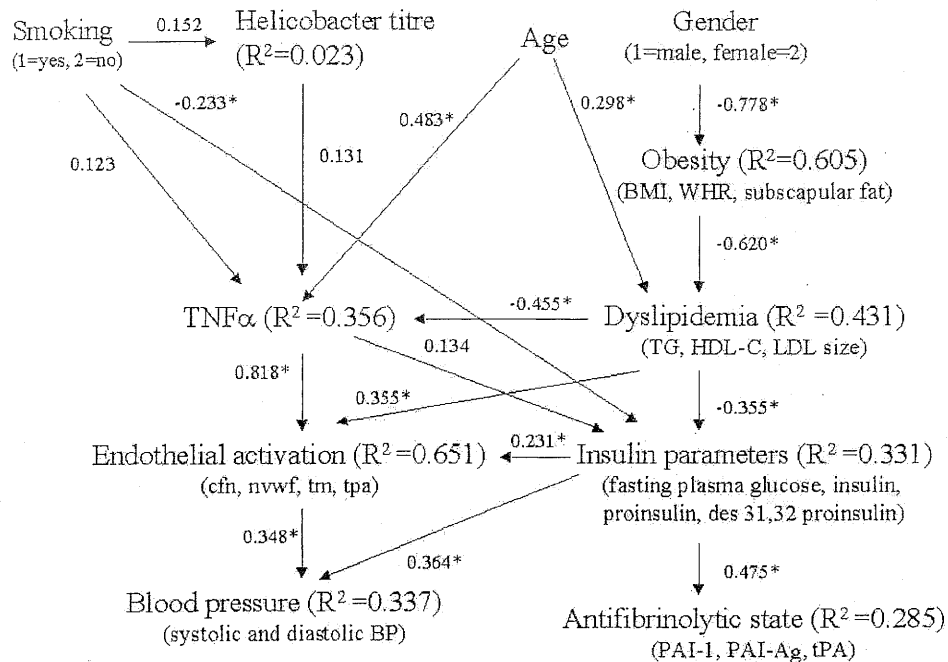
**Figure 2** The complex inter-relationships amongst the components of the metabolic syndrome were analysed by structural equation modelling using interleukin 6 (IL-6) as the main cytokine. The variance ( $r^2$ ) of each factor was explained by its relationships with other factors, which may interact amongst themselves. The strength of the relationship between two factors is indicated by the path coefficients, the significance of which is indicated by an asterisk ( $*P < 0.05$ ). Some of the paths which were consistent with theory but did not reach statistical significance were included while paths which did not have theoretical background were excluded. The model has a goodness of fit index of 0.76 and low root mean square residuals of 0.119, suggesting reasonable fit of these data to the proposed model. In this model, age, obesity-associated dyslipidaemia and cytokines were the main explanatory variables for hyperinsulinaemia and endothelial activation. The latter two factors interact to give rise to high blood pressure and an anti-fibrinolytic state. The following table complements the figure and shows the 95% confidence intervals of the causal path estimates.

Predictor	Outcome	Beta	t-Value	95% lower	95% upper
Helicobacter titre	Cytokine (interleukin-6)	0.183	2.101	0.039	0.327
Obesity	Dyslipidaemia	-0.685	5.157	-0.466	-0.904
Dyslipidaemia	Insulin	-0.403	3.145	-0.192	-0.614
Dyslipidaemia	Endothelial activation	0.283	1.867	0.033	0.533
Dyslipidaemia	Cytokine (interleukin-6)	-0.213	2.344	-0.063	-0.363
Insulin	Anti-fibrinolytic state	0.443	3.461	0.232	0.654
Insulin	Blood pressure	0.289	2.232	0.075	0.503
Insulin	Endothelial activation	0.604	3.088	0.281	0.927
Endothelial activation	Blood pressure	0.354	2.421	0.113	0.595
Cytokine (interleukin-6)	Insulin	0.038	0.433	-0.107	0.183
Cytokine (interleukin-6)	Endothelial activation	0.375	3.125	0.177	0.573
Smoking	Helicobacter titre	0.152	1.573	-0.007	0.311
Smoking	Insulin	-0.245	2.701	-0.095	-0.395
Smoking	Endothelial activation	-0.038	0.324	0.156	-0.232
Smoking	Cytokine (interleukin-6)	0.299	3.387	0.153	0.445
Age	Dyslipidaemia	0.311	3.716	0.173	0.449
Age	Cytokine (interleukin-6)	0.341	3.791	0.193	0.489
Female	Obesity	-0.769	7.664	-0.603	-0.935

between endothelial activation and the anti-fibrinolytic state, our findings regarding the intimate relationships between hyperinsulinaemia, endothelial activation and the anti-fibrinolytic state are all consistent with the current evidence.

**Unexpected effects of age, smoking and dyslipidaemia.** Although we were able to confirm most of the

hypothesized relationships amongst these interacting variables based on experimental or clinical evidence, we have identified paths which are hypothesis-generating and require confirmation (Figure 1b). Firstly, we found that age had an independent effect on dyslipidaemia and cytokines, which was not accounted by obesity. It remains plausible that age-related degenerative changes and increasing number of infections might be associated with increased production



**Figure 3** The complex inter-relationships amongst the components of the metabolic syndrome were analysed by structural equation modelling using tumor necrosis factor (TNF $\alpha$ ) as the main cytokine. The variance ( $r^2$ ) of each factor was explained by its relationships with other factors, which may interact amongst themselves. The strength of the relationship between two factors is indicated by the path coefficients, the significance of which is indicated by an asterisk (\* $P < 0.05$ ). Some of the paths which were consistent with theory but did not reach statistical significance were included while paths which did not have theoretical background were excluded. The model has a goodness of fit index of 0.75 and low root mean square residuals of 0.121, suggesting reasonable fit of these data to the proposed model. In this model, age, obesity-associated dyslipidaemia and cytokines were the main explanatory variables for hyperinsulinaemia and endothelial activation. The latter two factors interact to give rise to high blood pressure and an anti-fibrinolytic state. The following table complements the figure and shows the 95% confidence intervals of the causal path estimates.

Predictor	Outcome	Beta	t-Value	95% lower	95% upper
Helicobacter titre	Cytokine (TNF $\alpha$ )	0.131	1.684	0.003	0.259
Obesity	Dyslipidaemia	-0.620	5.483	-0.433	-0.807
Dyslipidaemia	Insulin	-0.355	3.223	-0.173	-0.537
Dyslipidaemia	Endothelial activation	0.355	2.981	0.159	0.551
Dyslipidaemia	Cytokine (TNF $\alpha$ )	-0.455	5.593	-0.321	-0.589
Insulin	Anti-fibrinolytic state	0.475	3.708	0.264	0.686
Insulin	Blood pressure	0.364	2.989	0.163	0.565
Insulin	Endothelial activation	0.231	1.708	0.008	0.454
Endothelial activation	Blood pressure	0.348	2.687	0.134	0.562
Cytokine (TNF $\alpha$ )	Insulin	0.134	1.454	-0.018	0.286
Cytokine (TNF $\alpha$ )	Endothelial activation	0.818	5.090	0.553	1.083
Smoking	Helicobacter titre	0.152	1.572	-0.008	0.312
Smoking	Insulin	-0.233	2.697	-0.090	-0.376
Smoking	Endothelial activation	-0.132	1.404	0.023	-0.287
Smoking	Cytokine (TNF $\alpha$ )	0.123	1.555	-0.008	0.254
Age	Dyslipidaemia	0.298	3.925	0.173	0.423
Age	Cytokine (TNF $\alpha$ )	0.483	5.981	0.350	0.616
Female	Obesity	-0.778	7.646	-0.610	-0.946

of inflammatory markers. Alternatively, dietary factors or hormonal changes, which may have an effect on lipid metabolism not measured in the present study, might have contributed to these relationships. On the other hand, while we were unable to show a direct relationship between obesity and cytokines, we found a strong and independent effect of dyslipidaemia on

cytokines. The nature of such association requires further exploration.

In our models, while smoking was a determinant for IL6, somewhat unexpectedly, it was associated with low levels of insulin products. The predictive role of smoking in the development of glucose intolerance has been reported but the exact mechanisms remain unclear.<sup>84-86</sup> Two recent

**Table 4** Lambda values for the loading variables in the confirmatory factor analysis using TNF $\alpha$  or IL6 as the cytokine parameters in separate models using a database of 107 Caucasian non-diabetic subjects. A lambda value greater than 0.3 suggests significant loading of the composite variables to the factors. An  $\alpha$  coefficient exceeding 0.7 suggests that the factor has good reliability

Factors	Composite measures	TNF $\alpha$ model		IL6 model	
		Lambda value	$\alpha$ coefficient	Lambda value	$\alpha$ coefficient
Blood pressure	Systolic blood pressure	0.889	0.805	0.937	0.811
	Diastolic blood pressure	0.748		0.704	
Obesity index			0.735		0.733
	Body mass index	0.425		0.427	
	Waist-hip ratio	0.898		0.896	
	Subscapular-triceps skinfold ratio	0.716		0.713	
Dyslipidaemia			0.736		0.729
	Triglyceride	-0.456		-0.475	
	HDL-cholesterol	0.489		0.552	
Insulin parameters			0.768		0.767
	LDL-particle size	1.057		0.984	
	Fasting plasma glucose	0.325		0.328	
	Fasting insulin	0.806		0.803	
	Fasting proinsulin	0.777		0.776	
Endothelial activation			0.643		0.563
	Fasting des 31,32 proinsulin	0.730		0.729	
	Cellular fibronectin	0.557		0.534	
	Von Willebrand factor	0.671		0.440	
	Thrombomodulin	0.517		0.336	
Antifibrinolytic state			0.752		0.741
	Tissue plasminogen activator	0.479		0.651	
	Plasminogen activator inhibitor-1 (PAI-1) activity	0.709		0.696	
	Plasminogen activator inhibitor-1 (PAI-1) antigen	0.923		0.956	
	Tissue specific plasminogen activator	0.457		0.388	

population-based studies have shown that low insulin secretion rather than insulin resistance was associated with smoking.<sup>86,87</sup> Together with its effects on cytokine production,<sup>71,88</sup> which can influence insulin action, the pathogenic mechanisms of smoking on glucose intolerance appear to be multifaceted and require further exploration.

**Summary.** In this pilot study, we have used a complex statistical method, albeit standard in many non-medical disciplines, to test a hypothesis regarding the linking roles of dyslipidaemia, cytokines and endothelial activation in the diverse clinical manifestations of the metabolic syndrome. We were able to confirm the intimate relationships between obesity, dyslipidaemia and hyperinsulinaemia, the effects of low grade infection on cytokines and that of cytokines on endothelial activation, the differences in the determinants and effects of IL6 and TNF $\alpha$ , as well as the effects of hyperinsulinaemia and endothelial activation on blood pressure and antifibrinolytic state (Figure 1b). Compared with conventional regression analysis, the explained variance of factors within these models was considerably higher, ranging from 20 to 60%. The path coefficients representing the strength of relationships between these factors were also significant and strong. Hence, our causal model examined by structural equation modelling is sustainable and the modelling also illustrates its potential value in biomedical research.

**Limitations.** However, there are certain caveats in the interpretation of results from these models. As discussed previously, we were unable to confirm the effect of age on obesity, that of obesity on cytokines as well as that of endothelial activation on the antifibrinolytic state. During the fitting of the model, we also had to include new paths, namely the effects of age on dyslipidaemia and cytokines, the negative effects of smoking on insulin levels as well as the effects of dyslipidaemia on cytokines. Although there are some data in the literature to support these relationships, further studies are required to evaluate their generality. Overall, the fitness of the data to the model was only fair since, ideally, the GFI index should exceed 0.9 when it was only 0.75. There are several reasons for this less than perfect fit. The cross-sectional nature of the survey may not fully address the potential temporal relationships of the hypothesized paths. The small sample size of this relatively low risk community-based population, the validity of various measurements to reflect true biological activities as well as the lack of measurements of important factors such as genetic, hormonal and psychosocial parameters may also reduce the goodness of fit of the final model.

Although structural equation modelling is an advanced and widely used statistical tool, it does have certain limitations. At present, there is no recommended method for power analysis to estimate the appropriate sample size to test a hypothesis using this modelling technique. Generally

speaking, a sample size 10 times that of the number of measured variables is often recommended. Hence, our present study which involves just over 100 subjects but in whom at least 30 variables have been measured can only be viewed as a pilot study which requires confirmation in large scale prospective studies. Furthermore, apart from derivation from theory and existing evidence, there are as yet no specific criteria for the omission or inclusion of a path or factor and hence, the whole context of the hypothesis has to be taken into consideration during the refinement of the model. Despite minor modifications, the objective of structural equation modelling remains to confirm or refute a predetermined hypothesis using both scientific evidence and exploratory data analysis to arrive at a final model which should be theoretically and statistically sound.

### Conclusions

Our present findings represent an extension to our previous analysis using the same methodology in another cohort in which obesity, age and family history explained the variance of most of the components of the metabolic syndrome.<sup>25</sup> Overall, our findings challenge the existing hypothesis regarding the central roles of insulin resistance and provide a more comprehensive explanation for the diverse clinical manifestations of this syndrome. Despite some limitations, in light of the plausible nature of our model, which has theoretical support, logical sequence and mathematical validity, we suggest that structural equation modelling is a useful research tool, in addition to experimental and cohort studies, which can be used by biomedical researchers to discover new knowledge and explain complex phenomena.

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