The influence of a Mediterranean Diet with and without Red Wine on the Haemostatic and Inflammatory Parameters of Subjects with the Metabolic Syndrome

E.P.G. Mansvelt¹, E. Fourie², D. Blackhurst³, T. Kotze¹, H. Stofberg², S. van der Merwe⁴, M.J. Kotze⁵, D. P. van Velden¹

(1) Department of Pathology, Haematology, Faculty of Health Sciences, University of Stellenbosch, PO Box 19063, Tygerberg, 7505, South Africa, epgm@sun.ac.za
(2) Haematology Laboratory, National Health Laboratories Service, Tygerberg Hospital, Tygerberg, South Africa
(3) Lipid Research Laboratory, Chris Barnard Building, Faculty of Health Sciences, University of Cape Town, Observatory, 7925, South Africa
(4) Department of Human Nutrition, Provincial Administration of the Western Cape, Tygerberg Hospital, Tygerberg, 7505, South Africa
(5) Genecare Molecular Genetics (Pty) Ltd., Christiaan Barnard Memorial Hospital, Cape Town, 8001, South Africa

Submitted for publication: January 2007
Accepted for publication: May 2007

Keywords: Mediterranean diet, wine, metabolic syndrome, haemostasis, inflammation, anti-oxidant, genotype

This 8 week study examined whether a Mediterranean diet supplemented with red wine, had an acute impact on subjects diagnosed with the metabolic syndrome. Twelve non-smoking subjects with diagnostic criteria of the metabolic syndrome on minimal medication, consumed a Mediterranean-like diet for 4 weeks respectively without and with red wine. The amount of red wine consumed was 250 ml (26 grams of alcohol) per day for male and 180 ml (19 grams) per day for female participants. A nutrigenetic profile for cardiovascular risk factors was performed on each participant. Fasting blood specimens were taken at baseline, after the diet and after the diet with wine interventions for platelet function, procoagulants FVII and FVIII, von Willebrand's factor, fibrinogen, tissue plasminogen activator, plasminogen activator inhibitor-1, highly sensitive C-reactive protein and oxygen radical absorbance capacity (ORAC). After both periods of diet without wine and with wine, ORAC increased significantly compared to baseline levels. Except for platelet H₂O₂ fluxes and FVII concentration, none of the haemostatic or inflammatory parameters changed significantly after the intervention periods compared with baseline levels. Genetic risk factors for cardiovascular disease were identified in all study participants and the potential genotypic effects relevant to this study were generally in agreement with expected phenotypic response following the dietary intervention. Our conclusions are that the period of intervention was too short for substantial changes in haemostatic or inflammatory parameters in subjects who already manifest some changes in their cardiovascular system and who showed diverse genetic profiles underlying increased cardiovascular risk.

INTRODUCTION

The metabolic syndrome is a cluster of clinical and metabolic abnormalities first described by Reaven (1988). Subjects with the metabolic syndrome have an increased risk of developing type 2 diabetes mellitus, cardiovascular disease and thrombosis (Grundy et al. 2004). Both genetic and environmental factors are involved in developing the syndrome.

It is now recognised that a pro-inflammatory state and endothelial dysfunction underlie the changes that promote atherosclerosis and cardiovascular disease in subjects with the metabolic syndrome. Levels of inflammatory markers such as C-reactive protein (CRP), cytokines such as interleukin 6 (IL6), tumour necrosis factor-α (TNF-α), levels of procoagulants for example fibrinogen and fibrinolytic inhibitors for example plasminogen activator inhibitor (PAI-1) are chronically increased in the plasma of subjects with the metabolic syndrome, reflecting the inflammatory and prothrombotic milieu.

Highly sensitive C-reactive protein (HS-CRP) and fibrinogen are markers of vascular inflammation in conditions such as atherosclerosis and the metabolic syndrome. Their raised levels indicate endothelial damage (Libby et al. 2002; Ridker et al., 2001). CRP is also produced in response to release of cytokines (IL-6, TNF-α) by the the visceral fat where a low grade inflammatory response exists in subjects with the metabolic syndrome (Yudkin et al. 1999). The MONICA study recently analysed the relationship between the levels of markers of systemic inflammation associated with cardiovascular disease and overall alcohol intake. The levels of markers such as fibrinogen and CRP were found to be significantly lower in moderate alcohol users compared to non-drinkers and heavy drinkers, suggesting that ethanol itself might have an anti-inflammatory effect (Imhof et al. 2004).

Platelets play an important role in thrombosis and in myocardial infarction (Ross, 1999). Platelets in essential hypertension show an increased tendency to aggregate (Nityanand et al. 1993) due to platelet activation under conditions of increased shear force and endothelial dysfunction (Blann et al. 2003). Platelets of diabetics are more aggregable than those of normal subjects (Tomaselli, 1990) and are more resistant to the antithrombotic effects of aspirin due to several factors, for example insulin resis-
Mediterranean diet effects on haemostasis and inflammation in the metabolic syndrome

Mediterranean diet as opposed to a control prudent diet. Moderate alcohol consumption has been shown to improve insulin sensitivity and to lower blood glucose levels (Howard et al. 2004). The ATTICA study reported a more favourable plasma profile of inflammatory and haemostatic markers in subjects who consumed a diet that most closely resembled a Mediterranean diet that included moderate wine consumption, compared to subjects who followed a Western type diet (Chrysohoou et al. 2004). In a controlled prospective intervention study on two groups of healthy volunteers, it was shown that the Mediterranean diet complemented by moderate red wine intake has favourable antithrombotic effects compared to a high fat diet complemented by red wine (Mezzano et al., 2001). The effects of alcohol and of wine on platelet function have been extensively researched. The findings of these studies were recently reviewed by Ruf (2004) and generally report an antithrombotic effect of moderate red wine consumption on platelet function.

Procoagulants and anticoagulants also participate in thrombogenesis: Factor VII (FVII) levels are affected by dietary triglycerides (Miller et al. 1986). The levels of Factor VIII (FVIII), fibrinogen and von Willebrand’s factor (vWF) are all affected by inflammatory responses (Morishita et al. 1996). The latter is also used as a marker of endothelial function (Blann and Taberner, 1995).

From a genetic perspective, an increased level of fibrinogen is associated with the β-fibrinogen polymorphism, β-fib, 455G→A in the β-chain of fibrinogen. The A-allele promotes a strong acute phase response. Homozygotes have a 7-10% higher level of fibrinogen with an increased rate of basal transcription (Van’t Hooft et al. 1999). An increase in 1 g/L fibrinogen increases the relative risk for coronary heart disease by 1.8 with added risk for individuals with diabetes or hypercholesterolaemia.

Glycoprotein Ib/IIa (GPIIa) is the platelet surface receptor for fibrinogen binding. The common 1565T→C polymorphism in exon 2 of the GPIIa gene is associated with increased platelet aggregability and myocardial infarction (Weiss et al. 1996). This Leu33Pro amino acid substitution (PII2 or HPA-1b) results in a conformational change in the amino-terminal disulfide loop involved in fibrinogen binding (Honda et al. 1995) and highlights the important role of increased platelet activation in the development of vascular disease (Cassar et al. 2003; Cherian et al. 2003).

Finally the fibrinolytic pathway’s efficiency to resolve a formed thrombus, plays an important role via tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) activities. Although the fibrinolytic inhibitor, PAI-1 is not considered an independent cardiovascular disease risk marker, elevated levels are associated with increased risk for arterial disease, thrombosis, obesity, metabolic syndrome, insulin resistance, type II diabetes and correlate with smoking and hypertriglyceridaemia (Loskutoff et al. 2000). The 4G/5G insertion-deletion polymorphism at nucleotide position –675 upstream of the PAI-1 gene affects gene transcription and the highest levels are found with two copies of the 4G allele (Panahloo et al. 1995). Presence of the 4G allele increases the risk of obesity 2 to 3-fold. Variable results for these fibrinolytic markers in relation to wine and/or alcohol consumption were described before, reviewed by Rotondo et al. (2000).

OBJECTIVES

The aim of our study was to evaluate the added effect of moderate red wine consumption to the Mediterranean-like diet on cardiovascular risk factors in subjects with the metabolic syndrome. The end points were the observed changes in risk factors (thrombotic and inflammatory parameters) after 4 weeks of the diet and after 4 weeks of the diet plus wine intervention. The response to intervention was also related to the genotype of the participants in an attempt to assess the potential impact of gene-diet interaction.

MATERIALS AND METHODS

Twelve non-smoking subjects aged 32 to 60 years on minimal medication, were selected by exhibiting 2 or more diagnostic criteria of the metabolic syndrome: central obesity (waist circumference >102 cm, males; >88 cm, females); increased blood pressure (>130 mmHg systolic; >85 mmHg diastolic); increased fasting blood glucose (>6.1 mmol/L) (Adult Treatment Panel III, 2001). They consumed a Mediterranean-like diet for 4 weeks without and with red wine, respectively. During the experimental periods the subjects increased their intake of vegetables, cereals, fruit, mono-unsaturated fatty acids (in the form of olive and canola oils) and fish at the expense of red meats and dairy products. Clear dietary guidelines were provided in the form of a lecture and instruction booklet. Patients were motivated to include 20 – 30 minutes of mild exercise (walking) into their daily routine. Dietary control was through 3 times 3-day dietary records at the end of the 2 week abstention from alcohol period preceding the interventions and during the third week of each intervention period. The amount of red wine added to the diet was 250 ml (26 grams of alcohol) per day for male and 180 ml (19 grams) per day for female participants. A genetic profile for cardiovascular risk factors was obtained for all the subjects to evaluate the potential impact of genetic risk factors on response to intervention. Genetic testing combined with medical and nutrition therapy has the potential to translate into effective risk reduction intervention (Fisler and Warden, 2005; Kotze and Badenhorst, 2005). Biochemical and haemostatic parameters that reflect the risk factors for cardiovascular disease, were determined on all participants to compare the effect of their habitual and newly acquired lifestyles and diet with and without wine. Fasting blood samples were taken at baseline, after 4 weeks of diet and after another 4 weeks of diet plus moderate red wine consumption. Weight, waist circumference and blood pressure were measured and the dietary records collected and checked by the dietician at each visit. Biochemical and physical results are reported elsewhere in this issue (van Velden et al., 2007).

Haemostatic and inflammatory parameters

Platelet aggregation and lag time on stimulation with collagen 1 and 5 mg/ml were determined on platelet rich plasma in a Coulter Chronolog aggregometer with Agrolink software (Beckman-Coulter) according to a standardized method (Yardu-
mian et al. 1986). As control, aggregation in response to arachidonic acid 75 µM was tested on all participants at every visit. Plasma thromboxane B2 (TxB2) was determined by a standard EIA method (Amersham).

Platelet activation was determined by flow cytometry as platelet H2O2 fluxes linked to stimulation of platelets by collagen according to the method of Del Principe et al. (1991). Catalase was used as inhibitor and to simulate the quenching role of red wine phenolic compounds in some of the ex vivo assays. In these assays platelets suspended in autologous plasma were pre-incubated with catalase for 30 minutes in the dark before collagen addition and immediate assessment by flow cytometry.

The oxygen radical absorbance capacity (ORAC) assay of Ou et al. (2001) was used to measure the anti-oxidant effect of the diet and wine intervention respectively on the plasma after wine and/or diet and compared to base line values. EDTA plasma was separated and frozen immediately after venepuncture to prevent oxidation.

Fibrinolytic parameters, tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) were determined by standardized EIA methods (Asserachrome, Stago); haemostatic parameters FVII and FVIII were determined by the one-stage factor assay using factor VII and FVIII deficient plasmas (Dade Behring) according to standardised methods (Laflan and Bradshaw, 1995). von Willebrand’s factor (vWF) was determined by a latex enhanced immunoassay on the ACL 9000 (Instrumentation Laboratory).

Inflammatory markers were particularly high in some of the participants. Neither CRP nor fibrinogen levels changed significantly after any of the interventions. There was a direct correlation between fibrinogen and CRP levels at baseline (r = 0.82, p < 0.01) but after adjustment the correlation was no longer significant (Fig. 2A). There was also a direct correlation between fibrinogen and CRP levels after wine with diet (r = 0.87, p < 0.001 that remained significant after adjustment (r = 0.66, p < 0.001 that remained significant after adjustment (r = 0.66,
Coagulation and fibrinolytic parameters

Procoagulants FVIII, vWF and fibrinolytic parameters tPA and PAI-1 levels did not change significantly after any of the interventions. FVII levels increased after the intervention periods, significantly after diet compared to baseline (p < 0.05) (Fig. 3). PAI-1 levels were in the upper normal range (normal range 4-43 ng/ml) and increased after wine consumption. Results are given in Table 1.

Antioxidant parameters

The Mediterranean-like diet without wine had a protective antioxidant effect in the plasma. There was a statistically significant increase in the ORAC after the 4 week diet intervention period (Fig. 4), from 7.5 ± 1.4 mmol/L trolox equivalents (TE) to 9.3 ± 2.5 mmol/L TE, (p = 0.03). This may be attributed to the olive oil-based, alpha-linolenic acid-rich, Mediterranean-type diet. The ORAC decreased slightly when wine was added to the diet, to 8.5 ± 1.1 mmol/L TE, but this was not statistically significant (p = 0.342), and the increase in the ORAC was still significantly different from the baseline concentration (p = 0.04).

When the ORAC levels were correlated with the other parameters, a significant negative correlation (r = -0.67, p < 0.05) was found with CRP only after the 4 week wine and diet intervention period (Fig. 5). This finding suggests an anti-inflammatory effect of anti-oxidants in red wine. A similar trend was found with fibrinogen (r = -0.56, p = 0.06). No significant correlations were found between ORAC levels and the other haemostatic inflammatory markers (vWF, FVIII or PAI-1).

TABLE 1
Results of platelet function, haemostatic and inflammatory tests on 12 participants at baseline, after diet and after wine plus diet interventions given as mean ± 2 SD.

<table>
<thead>
<tr>
<th>Test</th>
<th>Baseline</th>
<th>Diet</th>
<th>Wine + Diet</th>
<th>*p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Aggreg Coll 1</td>
<td>56.67±10.6</td>
<td>73.17±6.1</td>
<td>64.08±7.4</td>
<td>0.27</td>
</tr>
<tr>
<td>Coll 1 lag sec</td>
<td>73.42±7.85</td>
<td>74.6±5.58</td>
<td>79.08±8.3</td>
<td>0.56</td>
</tr>
<tr>
<td>%Aggreg Coll 5</td>
<td>73.67±12.5</td>
<td>82.0±6.12</td>
<td>76.33±8.94</td>
<td>0.715</td>
</tr>
<tr>
<td>Coll 5 lag sec</td>
<td>42.17±2.13</td>
<td>42.17±2.72</td>
<td>44.08±2.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Fluor Coll+Cat</td>
<td>34.69±8.28</td>
<td>16.89±0.71</td>
<td>27.88±2.87</td>
<td>0.005</td>
</tr>
<tr>
<td>Fluor Coll</td>
<td>51.93±9.85</td>
<td>21.41±2.22</td>
<td>32.94±4.55</td>
<td>0.006</td>
</tr>
<tr>
<td>TxB2 pg/well</td>
<td>1.96±0.41</td>
<td>2.06±0.28</td>
<td>2.37±0.72</td>
<td>0.82</td>
</tr>
<tr>
<td>Fibrinogen g/L</td>
<td>4.84±0.31</td>
<td>4.83±0.37</td>
<td>4.57±0.45</td>
<td>0.52</td>
</tr>
<tr>
<td>FVII %</td>
<td>135.83±5.22</td>
<td>144.42±6.06</td>
<td>147.83±5.68</td>
<td>0.031</td>
</tr>
<tr>
<td>FVIII %</td>
<td>110.08±15.09</td>
<td>121.83±15.54</td>
<td>118.25±15.48</td>
<td>0.028</td>
</tr>
<tr>
<td>VWF %</td>
<td>142.17±12.42</td>
<td>142.08±11.53</td>
<td>143.33±11.63</td>
<td>0.936</td>
</tr>
<tr>
<td>TPA ng/ml</td>
<td>6.33±0.87</td>
<td>6.51±0.99</td>
<td>6.74±0.7</td>
<td>0.861</td>
</tr>
<tr>
<td>PAI-1 ng/ml</td>
<td>53.52±12.04</td>
<td>57.96±8.63</td>
<td>65.83±12.24</td>
<td>0.372</td>
</tr>
<tr>
<td>ORAC mmol/L TE</td>
<td>7.45±0.40</td>
<td>9.31±0.714</td>
<td>8.52±0.32</td>
<td>0.043</td>
</tr>
<tr>
<td>CRP mg/L</td>
<td>6.29±1.97</td>
<td>6.91±2.73</td>
<td>6.64±2.43</td>
<td>0.733</td>
</tr>
</tbody>
</table>

*p-values reflect differences between the base line and the interventions. Abbreviations: Aggreg = aggregation; Coll = collagen; lag = lag time; Fluor = fluorescence; Cat = catalase.
Epidemiological studies that correlate alcohol intake with markers of inflammation found an inverse relation between the amount of alcohol consumed and markers like fibrinogen, white blood cell count and FVIII, except for CRP where the relationship was significantly modified for subjects with the ApoE4 genotype in subjects with no evidence for cardiovascular disease (Mukamal et al, 2004). In our study 4 of our subjects had the ApoE4 genotype and their mean CRP levels were higher initially and after both interventions than those of the ApoE4 negative subjects. The median levels however, did not differ between the groups (Table 2) and CRP levels varied widely in both groups.

The ATTICA study reported a more favourable plasma profile of inflammation and haemostasis markers (Chrysohoou et al. 2004) as well as significantly elevated total anti-oxidant capacity and lower oxidized LDL cholesterol levels in individuals who consumed a diet that most closely resemble a Mediterranean diet which included moderate wine consumption, compared to subjects who followed a Western type diet (Pitsavos et al. 2005). In our study the plasma anti-oxidant capacity measured by ORAC showed a significant increase after both the diet and the diet with wine intervention periods.

Imhof et al. (2004) found general anti-inflammatory effects for alcohol in the form of wine or beer on markers such as white blood cell count, plasma viscosity, CRP and fibrinogen in a report on the MONICA study. Contrary to the literature findings, red wine demonstrated no additional anti-inflammatory or anti-haemostatic effects in our subjects as evaluated by CRP, fibrinogen, vWF, FVIII, tPA and PAI-1 levels that did not change significantly after any of the interventions. Contrary to our expectations, FVII increased significantly after diet intervention compared to baseline levels which could not be explained by correlations with triglyceride or weight. Similar results were also found in another study, where addition of red wine did not add to the benefits of a Mediterranean-like diet on the haemostatic factors except for prolongation of the bleeding times (Mezzano et al. 2003). Another confounding finding was the significant correlation between CRP and fibrinogen after the diet with wine period, implying that the diet without the wine has an attenuating effect on the inflammatory markers while the addition of wine did not.

Our study investigated the short-term beneficial effects of moderate wine consumption on biological parameters. It could be that the effect of diet and wine cannot be demonstrated in such a short intervention period. It is also probable that the diet showed more favourable effects on health parameters such as the anti-oxidant capacity of the plasma because the diet was continued for double the period of time as for that of wine intake. Wine was added last in the intervention, however, and the beneficial anti-oxidant effect of the diet alone could have been partially reversed by the addi-
tion of alcohol that acted pro-oxidatively in spite of our red wine’s high phenolic content. The inverse relationship between ORAC and CRP levels that reached significance after the diet plus wine period (Fig. 5) suggests instead that the addition of red wine-derived phenolics to the diet positively contributed towards an anti-inflammatory effect.

**Genotype effects**

When considering the genetic risk factors implicated in the development of arterial disease or thrombosis related to the β-fibrinogen 455G→A, PAI-1 –675 4G/5G and GPIIIa 1565T→C polymorphisms, the potential significance of combined genotypic effects was highlighted. Half of the participants had 2 of these 3 polymorphisms and 3 others had one of the 3 gene polymorphisms.

Three participants were heterozygous for the –455G→A polymorphism in the β-fibrinogen gene, while one was homozygous for the A-allele. Two of the heterozygotes had persistently high fibrinogen levels (>5.0 g/L) and in only one of these individuals did the fibrinogen levels decrease after the diet with wine period. The mean and median fibrinogen levels for the 4 participants with the β-fibrinogen polymorphism were higher at baseline, after diet and after diet and wine interventions compared to those of the other 9 participants at baseline, after diet and wine interventions. Their platelet aggregation responses as maximum amplitude were much higher than those of the 9 participants without the polymorphism.

When considering the genetic risk factors implicated in the development of arterial disease or thrombosis related to the β-fibrinogen 455G→A, PAI-1 –675 4G/5G and GPIIIa 1565T→C polymorphisms, the potential significance of combined genotypic effects was highlighted. Half of the participants had 2 out of these 3 polymorphisms and 3 others had one of the 3 gene polymorphisms.

Three of the study participants had the PI-1 or HPA-1b allele of the 1565T→C polymorphism in exon 2 of the GPIIIa gene of GPIb/IIIa platelet surface receptor for fibrinogen binding. When their platelet aggregation responses as maximum amplitude were compared with those of the other 9 participants at baseline, after diet and after diet with wine interventions, their mean and median platelet aggregation responses with 5 µg/ml collagen were much higher than those of the 9 participants without the polymorphism.

Eight of our study subjects had the 4G allele of the PAI-1 promoter polymorphism, of whom 3 were homozygous for this allele. PAI-1 levels did not differ between the 2 groups. Presence of the 4G allele carries a higher risk for overweight or obesity and may also explain the absence of response in the PAI-1 levels after the interventions.

Although the findings were limited by the small number of subjects studied over a relatively short period of time, some important observations were made. Notably, the presence of the mutated allele of the GPIIIa polymorphism seems to prevent platelet response to both interventions when compared to platelet response of the participants before and after interventions as evaluated by the platelet aggregation with 5 µg/ml collagen. Similar observations were made in relation to fibrinogen levels, as participants with the β-fibrinogen polymorphism maintained the increased fibrinogen levels after both interventions.

**CONCLUSIONS**

This short-term and intensive study included 12 participants with positive markers of the metabolic syndrome whose responses to intervention appeared to be affected by their genotype. The diet and wine interventions increased the anti-oxidant potential of the plasma and probably at intracellular level as reflected by the platelets. The markers of inflammation and haemostasis did not change significantly after the intervention periods due to the short duration of the study, the already existing pathology in our target subjects and the different reactions linked to specific genotypes as discussed.

For future studies we recommend longer intervention periods of diet with and without wine, a larger study population that allows for comparison of response to the interventions between larger genotypic subgroups.

**LITERATURE CITED**


