NEW PREDICTORS OF CORONARY CARDIOPATHY: CIRCULATING ENDOTHELIAL MICROPARTICLES, FUNCTIONALITY OF THE HDL PARTICLES AND COMPOSITION IN FATTY ACIDS OF THE CELLULAR MEMBRANES

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1. Summary of the original project

1. To establish novel and emergent predictors for coronary heart disease (CHD), in middle-aged and elderly subjects at high cardiovascular risk followed for 5 years; Different parameters will be studied related to:

1.1. HDL functionality (cholesterol efflux capacity, antioxidant, anti-inflammatory, endothelial protection, vasodilatory, and antiapoptotic action) and protein-lipid composition of HDL (amyloid serum A, C3 complement and A-IV and C-III apolipoproteins).

1.2. Fatty acid composition of whole blood cell membranes (targeted lipidomic approach).

1.3. Circulating endothelial microparticles.

2. To study the modulation promoted by the Mediterranean diet intervention on the incidence of CHD. The association of the CHD event and the changes of these predictors after one year of the different dietary interventions will be determined.

3. To determine the prognostic value of these novel biomarkers in the patients who have had a myocardial infarction.

Secondary objectives

1. To examine the relationship between the novel predictors of CHD related to baseline fatty acid composition of white blood cell membranes, HDL functionality, and endothelial microparticles, and a selection of circulating inflammatory markers.

2. To evaluate the usefulness of these novel biomarkers in determining the prognosis of patients with myocardial infarct.

2. Results

The objectives of the group coordinated by **Dr Estruch** were the determination of circulating endothelial microparticles (cMP) as potential biomarkers in prevention and treatment of coronary heart disease.

To determine the effects of the Mediterranean diet on the release of cMP to circulating blood, 150 samples were selected (50 per intervention group: extra-virgin olive oil (EVOO), nuts and control diet of the PREDIMED study) in patients without CVD at the end of the study. The following analyses were performed at baseline and one-year follow-up visit: endothelial cMP in peripheral blood plasma, platelets, leucocytes and erythrocytes by flow cytometry and fatty acids in plasma by gas chromatography according to the panel of antibodies shown below.

Panel of antibodies:

Cell origin	**Markers**		
Annexin control	-	-	Annexin V
Isotypes	IgG 1 γ	IgG 1 κ	
Platelets	PAC-1	CD62P	Annexin V
Platelets	CD142	CD61	Annexin V
Endothelial	CD146	CD62E	Annexin V
Erythrocytes	CD235ab	CD34	Annexin V
Leucocytes	CD3	CD45	Annexin V
Monocytes	CD11a	CD14	Annexin V
Smooth muscle cells	CD142	SMA-a	Annexin V
Activated cells	CD63	CD62L	Annexin V

After one year of dietary intervention, the concentration of cMP derived from platelets was lower in the groups supplemented with nuts compared with EVOO and the control diet. In addition, prothrombotic cMP (transporters of tissue factor and markers of cell activation) were reduced after one year on the Mediterranean diet (supplemented with both EVOO and nuts) compared to the control diet.

The functionality of circulating procoagulant microparticles was also studied. 132 subjects at high risk of CHD were selected, 66 of whom were part of the Mediterranean diet intervention group (supplemented with olive oil or nuts) and 66 were in the control group. We measured the procoagulant activity of the microparticles at baseline, 6 to 12 months of the intervention. These analyses were performed with the ZYMUPHEN MP-Activity functional test (ANIARA, HyphenBioMed) for functional determination of microparticles in plasma using a colorimetric method, which analyse the procoagulant capacity of circulating microparticles.

The results showed that the Mediterranean diet might have a protective effect on the coronary process. The microparticles showed no changes in coagulant function; their activity did not significantly vary at either 6 or 12 months. However, following a low-fat diet favoured an increase in the procoagulant activity of the microparticles, thereby indicating the absence of a protective effect that could trigger some types of coronary event.

Evaluation of inflammatory molecules and those related to plaque stability showed similar results. Thus, it was found that the group on the Mediterranean diet supplemented with olive oil or nuts presented a significant reduction in the plasma levels of MMP-9, endothelin, SST2, IL-6, IL-18, TNF-α and MCP-1 ($P<0.05$, all) and a significant increase in IL-10 ($P= 0.013$). MMP-9 is a molecule, which, similar to IL-18, is related to plaque instability, while IL-10 favours plaque stability. No changes in VCAM-1 or ICAM-1 concentrations were observed with either of the two diets. Therefore, the Mediterranean diet seems to have an immunomodulatory effect on biomarkers of cardiovascular risk such as SST2, IL-6, IL-10, IL-18, MMP-9, TNF-α and MCP-1.

Dr Fitó's group

They conducted a case-control study nested within the PREDIMED (Prevención con Dieta Mediterránea) cohort, originally a randomized trial where participants followed a Mediterranean or low-fat diet. Incident acute coronary syndrome cases (N=167) were individually matched (1:2) to controls by sex, age, intervention group, body mass index, and follow-up time. They investigated its two individual manifestations (myocardial infarction, unstable angina) as secondary outcomes. The following functional characteristics were measured: HDL cholesterol concentration (in plasma); cholesterol efflux capacity; antioxidant ability measured by the oxidative HDL inflammatory index; phospholipase A2 activity; and sphingosine-1-phosphate, apolipoproteins A-I and A-IV, serum amyloid A, and complement 3 protein (in apolipoprotein B-depleted plasma). Conditional logistic regression models adjusted for HDL cholesterol levels and cardiovascular risk factors were used in order to estimate odds ratios (ORs) between one standard deviation increments in HDL functional characteristics and clinical outcomes.

At present, they are writing the meta-analysis paper; they observed that higher cholesterol removal from macrophages prompted by apo B depleted in serum was associated with coronary events. Although the results from the selected studies are aligned with this statement, one study found opposite results. Only 9 articles were found meeting inclusion criteria for antioxidant capacity of HDL, of which 5 had data on the risk of all-cause mortality and 7 on the risk of coronary events. Out of the 9 manuscripts, 4 were case control studies and all studies were carried out in small population. This meta-analysis identified the need for larger longitudinal studies with standardized designs. However, experimental approaches along different studies were quite consistent, which increased comparability among results.

Dr Salas's group

During the last year of the project they conducted the remaining metabolomic analysis of cell membrane composition to cover a wide spectrum of different fatty acids. They also integrated all metabolomic profiles obtained during the analytical process and created the final data base with the inclusion of clinical data, metabolomic data, and data for inflammatory markers and HDL.

A last analysis was focused on the relationship between fatty acid cell membrane composition, HDL functionality, and inflammatory status. The results show an association between HDL functionality parameters and fatty acid cell membrane composition (Table 1).

Table 1: HDL functionality parameters ranked by elastic net positive or negative regression coefficients for cell membrane fatty acids

	ApoA-IV	CEC	HOII	S1P	SAA	ApoA-I	ApoC-III	C3 protein
C14:0	None	None	None	None	0.00927	0.01868	-0.14057	None
C16:0	None	None	None	None	None	None	0.622764	None
C16:1n7*trans*	None	None	None	None	0.18307	None	None	None
C16:1n7*cis*	None	None	-0.013	None	None	None	0.120216	None
C18:0	None	-0.0404	0.1098	-0.353	None	None	-0.99713	None
C18:1n9*cis*	None	None	None	0.13603	None	None	None	None
C18:1n9*trans*	None	None	None	-0.392	None	-0.0447	-1.27781	None
C18:2n6	None	-0.0294	None	None	None	0.11717	-0.10579	None
C18:3n3	None	-0.0178	None	-0.1138	-0.0899	None	0.00328	None
C20:0	None	None	None	None	-0.2632	None	-0.10936	None
C20:1n9	None	None	0.0601	None	None	None	None	None
C20:2n6	None	None	None	None	0.34364	None	None	None
C20:3n6	None	None	None	None	0.22568	None	None	None
C20:4n6	None	None	None	None	None	None	None	None
C20:5n3	None	None	None	None	None	None	-0.07868	None
C22:0	None	None	None	-0.021	None	None	None	None
C22:4n6	None	None	None	None	None	None	None	None
C22:5n6	None	None	None	None	-0.3201	None	None	None
C22:5n3	None	None	None	None	0.02681	None	None	None
C24:0	None	None	None	-0.0472	0.24229	None	None	None
C22:6n3	None	None	None	None	None	None	None	None
C24:1n9	None	None	None	None	None	0.00524	None	None
Total SFA	None	None	None	None	None	None	None	None
Total MUFA	None	None	None	None	None	None	0.240921	None
Total n-6 PUFA	None	None	None	None	None	None	None	None
Total n-3 PUFA	None	None	None	None	None	None	None	None
n6:n3 PUFA	None	None	None	None	None	None	None	None

Additionally, several HDL functionality parameters were related to inflammatory markers, mainly IL.6 (Table 2).

Table 2. HDL functionality parameters ranked by elastic net positive or negative regression coefficients for inflammatory markers (n=259)

	ApoA-IV	CEC	HOII	S1P	SAA	ApoA-I	ApoC-III	C3 protein
IFNγ	None	None	None	None	None	None	None	None
IL-1b	None	None	None	None	0.0184	-0.0651	None	None
IL-6	None	None	None	0.1545	0.1196	-0.1227	None	-0.0229
IL-8	None	None	None	None	None	None	None	None
IL-10	None	None	None	None	None	None	None	None

Adjusted for age, sex, body mass index, HDL levels at baseline, smoking, type 2 diabetes, and hypercholesterolemia.

Interestingly, the content of these molecules in HDL particles, mainly those related to IL-6, were also associated to specific fatty acids at cell membranes suggesting a potential role of cell membrane fatty acid composition on inflammation and HDL functionality (Table 3).

Table 3. Cell membrane fatty acids ranked by elastic net positive or negative regression coefficients for HDL functionality parameters, when both are associated with IL-6 (n=259)

Fatty acids	HDL structure/ functional parameters		
	S1P	SAA	ApoA-I
C14:0	None	None	0.0166
C20:0	-0.0097	None	None
C16:1n7*trans*	-0.0908	0.0843	None
C18:3n3	-0.0510	-0.0097	None

Adjusted for age, sex, body mass index, HDL levels at baseline, smoking, type 2 diabetes, and hypercholesterolemia.

3. Relevance to possible future implications

Prevention is the first step in the treatment of many diseases such as cardiovascular diseases. Using specific biomarkers for these diseases may improve the identification of subjects at high cardiovascular risk who do not fit the current risk classifications. Thus, one of the objectives of this project was to validate the utility of three new possible biomarkers (HDL functionality, composition of fatty acids of the cellular membranes and circulating endothelial microparticles) in the prediction of new coronary heart disease events in subjects at high risk but asymptomatic (primary prevention) and in patients who have had myocardial infarction (secondary prevention). The results obtained are totally innovative since they combine biomarkers, dietetic food patterns and clinical data and may be of maximum interest to the medical community. The information on the prognostic value of these biomarkers in patients who have had a heart attack is absolutely new. In addition, to date, the methodology of the circulation of endothelial microparticles has never been used in food intervention studies.

On the one hand, the results obtained by Dr. Fitó's group are very promising as these might be incorporated with the measurement of some of these biomarkers (e.g., those related to HDL composition such as levels of ApoA-I or S1P in apolipoprotein B-depleted plasma samples) as biomarkers for clinical management and ACS prognostic tool for high-risk subjects. This suggestion is feasible because: 1) apolipoprotein B-depleted plasma samples are easy to obtain (its collection only takes 30 minutes and requires cheap reagents and standard laboratory equipment) (Rohatgi A, N Engl J Med, 2014); and 2) quantitative techniques are easy to standardize and implement in routine laboratories.

On the other hand, Dr. Salas's results have demonstrated that higher levels of specific fatty acids in the cell membrane, such as C:22 and C:24 are associated with a lower CHD incidence, whereas higher levels of C20:1n9 are associated with higher risk. We have also demonstrated that differences in the composition of fatty acid blood cell membrane are associated with serum levels of different inflammatory markers and consistently, 1-year changes in blood cell fatty acids levels were associated with changes in these inflammatory markers, supporting a potential preventive and/or therapeutic strategy to fight against inflammatory-related diseases.

The results obtained by Dr Estruch's research group have also shown that the consumption of certain fatty acids in the context of a Mediterranean diet reduce the levels of cMPs from different cellular origins. In addition, the procoagulant activity of the microparticles may be halted by the action of the diet itself, contrary to what would happen with a low-fat diet, which seems to significantly increase the procoagulant action of the microparticles.

Although our results (Dr. Salas and Dr Estruch) contribute to open new research avenues in the field of CHD and cardiovascular disorders, our results do not have immediate applicability in health care. Future steps should not only confirm our findings in other populations but also elucidate how cell membrane composition modifies cell activity and whether cell membrane fatty acid composition could be modified to achieve a healthy cell, and on the other hand, to see how the diet is capable of modulating the procoagulant capacity of the circulating microparticles. Nevertheless, the results of this project could improve the treatments and clinical decisions towards patients with acute coronary syndrome and may affect the information for decision-making or administrative policies and, consequently, improving the quality of life or the satisfaction of patients with cardiovascular diseases. Prevention of primary coronary heart diseases is still based on the Framingham risk scale, which has been used for more than 40 years. Thus, the combination of previous knowledge with the current possible biomarkers of risk is a perfect tool to obtain the 4Ps required from quality personalized medicine (predictive, preventive. personalized and participative).

4. Scientific bibliography generated

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