Combined bioactive approach over atherosclerosis risk biomarkers

Bianca Scolaro

São Paulo
2017
UNIVERSITY OF SÃO PAULO
Faculty of Pharmaceutical Sciences
Graduate Program in Food Sciences
Area of Experimental Nutrition

Combined bioactive approach over atherosclerosis risk biomarkers

Bianca Scolaro

Original Version

Ph. D. Thesis presented for the degree of
DOCTOR OF SCIENCE

Advisor: Prof. Dr. Inar Alves de Castro

São Paulo
2017
Scolaro, Bianca

S421c

COMBINED BIOACTIVE APPROACH OVER ATHEROSCLEROSIS RISK BIOMARKERS / Bianca Scolaro. - São Paulo, 2017:

92 p.

Tese (doutorado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Alimentos e Nutrição Experimental. Orientador: Alves de Castro, Inar

1. Dislipidemia. 2. Funcionalidade HDL. 3. Estatinas. 4. Ácidos graxos ômega 3. 5. Fitostórisis. 1. T. II. Alves de Castro, Inar, orientador.
Combined bioactive approach over atherosclerosis risk biomarkers

Commission of Thesis for the degree of Doctor of Science

1st Examiner

2nd Examiner

3rd Examiner

4th Examiner

5th Examiner

São Paulo, ____________________________, 2017.
Firstly, I would like to express my sincere gratitude to my advisor Prof. Inar Alves de Castro for her guidance and continuous support of my Ph.D study. I am grateful for her endless motivation and immense knowledge; for encouraging and allowing me to grow as a research scientist. Her advices, both on professional and life matters, have been priceless.

I would especially like to thank the staff at the Dante Pazzanese Institute of Cardiology, for the support while I recruited patients and collected data for my Ph.D. thesis. Most of all, my special thanks to Dr. Adriana Bertolami for all her expertise and assistance, that made the clinical study possible.

My sincere thanks also goes to Dr. Edward Fisher and Dr. Sean Heffron, who provided me an opportunity to join their team as visiting student at the New York University School of Medicine. I am thankful for the great mentorship, education and invaluable pep talks. This was an outstanding experience, for both my scientific and personal growth.

I also thank the Fisher lab members for their help and for making the internship so enjoyable, even when all experiments seemed to fail. Special thanks to Tatjana Josefs, Shruti Rawal and Sean Heffron (the “HDL team”); to the technicians Felix Zhou, Alexandra Quezada and Michela Garabedian for all the assistance and laughter; to Jaume Amengual, Karishma Rahman and Beyza Vuruşaner for the stimulating discussions and advices; and to Ada Weinstock, and her husband Felix Ilie, not only for the scientific help, but also for their invaluable friendship, for being our family from far away, and for the funniest version of “A Tonga da Mironga do Kabuletê” we have ever heard. I also place on record, my deep gratitude to Milessa Afonso, from the lab across the hall, for being the Brazilian friend that would always understand me; some feelings can only be expressed in Portuguese.

I wish to express my sincerest gratitude and warm appreciation to all my fellow labmates who came and went during the years, for always working together as a team, helping me to process my samples, and for all the fun we have had in the last four years. In particular, I am grateful to Marina Nogueira, for always trying to do whatever she could to help me in any possible way and for being such a good friend; to Helena Souto for all her help and friendship; and to Livia Chassot and
Gabriela Grassmann Roschel for all the great moments in and outside the lab. I must also put on record, my appreciation for a number of friends from different labs who much contributed to my project in all sorts of ways, or by simply being there for me: Mayara Miranda, Raquel Cruz, Mariana Rosim, Camile Fontelles, Luiza Guido, Fabia Andrade, Lívia Ribeiro, Natália Castro, Gabriela Costa e Vanessa Cardoso Pires.

A special thanks to my family: to my loving parents, brother and sister, and my in-laws, for their encouragement and for supporting me spiritually throughout the years.

Last but not the least, I would like to thank my beloved husband Gregory Thom e Silva, for being there to bring me up whenever life puts me down; for always doing the impossible to align our careers so that we can pursue our dreams together; for sharing your passion for science with me and never letting me give up on my love for science too.

This work would not have been possible without the financial support of CNPq (163211/2013-2), CAPES and FAPESP (FAPESP grants 14/04247-3, 15/16243-5, 15/18859-3), and without the commitment from all the employees from the Food and Experimental Nutrition Department towards all students.
“I am among those who think that science has great beauty”

Marie Curie
RESUMO

SCOLARO, B. Abordagem combinada de compostos bioativos sobre biomarcadores de risco para aterosclerose. 2017. 92f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2017.

A aterosclerose, uma importante causa mundial de morbidade e mortalidade, é uma doença complexa e multifatorial que envolve três princípios condições: inflamação crônica, dislipidemia e estresse oxidativo. Embora as estatinas sejam fármacos de primeira linha para redução de LDL colesterol (LDL-C), sua eficácia na prevenção de eventos cardiovasculares é limitada a 30-40%. Este risco cardiovascular residual evidencia a necessidade de novas terapias e marcadores clínicos que vão além do LDL-C, como inflamação e estresse oxidativo. Não obstante, tratamento subótimos e/ou interrupção do uso de estatinas devido à ocorrência de efeitos adversos também é um grave obstáculo na clínica médica. Neste contexto, a terapia dietética complementar representa uma abordagem efetiva e segura para o suporte do tratamento farmacológico, especialmente quando as drogas são insuficientes para atenuar fatores de risco e/ou quando a dose recomendada não é bem tolerada. O objetivo do presente estudo foi avaliar o efeito de três compostos bioativos – ácidos graxos ômega 3, fitosteróis e polifenóis – sobre marcadores de inflamação, lipemia e estresse oxidativo em indivíduos tradados com estatinas. Foi realizado um estudo clínico randomizado, de delineamento crossover, com a participação de 53 voluntários. A cada período de intervenção, os participantes receberam um tratamento funcional ou controle. O tratamento funcional foi composto por cápsulas de óleo de peixe (1.7 g/dia de EPA+DHA), chocolate contendo fitosteróis (2.2 g/dia) e chá verde (dois sachês/dia). O tratamento controle foi composto por cápsulas de óleo de soja, chocolate sem adição de fitosteróis e chá de anis. Após 6 semanas de intervenção, o tratamento funcional reduziu a concentração plasmática de LDL-C (-13.7% ± 3.7, p=0.002) e proteína C-reactiva (-35.5% ± 5.9, p=0.027). Triglicerídeos (-15.68% ± 5.94, p=0.02) e malondialdeído (-40.98% ± 6.74, p=0.04) foram reduzidas apenas em subgrosos de indivíduos que apresentavam valores basais acima da media (93 mg/dL e 2.23 umol/L, respectivamente). A análise de latosterol e campesterol no plasma sugeriu que a intensidade da redução de LDL-C não foi influenciada pela síntese endógena de colesterol, mas sim pela taxa de absorção. Após análise multivariada dos resultados, pacientes identificados como “good responders” à suplementação (n=10) foram recrutados para um estudo piloto de redução da dosagem da estatina, aliado à terapia dietética complementar. Estes pacientes receberam o tratamento funcional por 12 semanas: durante as 6 primeiras semanas manteve-se a dosagem de estatina, que em seguida foi reduzida em 50% das semanas 6 a 12. Não foram observadas diferenças para os marcadores plasmáticos de lipídeos, inflamação, capacidade de efluxo de colesterol ou número de partículas de HDL após a redução da dose de estatina, quando comparada à terapia convencional. Embora limitado pelo reduzido número de pacientes, o estudo demonstra o potencial para uma nova abordagem terapêutica, combinando reduzida dose de estatina com específicos compostos bioativos. Esta pode ser uma importante alternaativa para muitos pacientes em risco cardiovascular e que são intolerantes à terapia com altas doses de estatina.

Palavras-chave: Dislipidemia, funcionalidade HDL, estatina, ácidos graxos ômega 3, fitosteróis, polifenóis
Atherosclerosis, one major cause of morbidity and mortality worldwide, is a complex and multifactorial disease that involves three mainly conditions: chronic inflammation, dyslipidemia and oxidative stress. Although statins are the first-line therapy for LDL cholesterol (LDL-C) lowering, the efficacy of cardiovascular events prevention is limited to 30-40%. This residual risk brought attention to the need of new therapies and clinical targets beyond LDL-C, such as inflammation and oxidative stress. Importantly, suboptimal treatment and/or statin discontinuation due to adverse effects have also been a very challenging clinical problem. Complementary diet therapy can be an effective and safe approach to support pharmacological treatment, especially when drugs alone are insufficient to attenuate risk factors and/or the recommended dose is not well tolerated. The aim of this study was to evaluate the effects of three bioactive components, namely omega-3 fatty acids, plant sterols and polyphenols, on markers of dyslipidemia, inflammation and oxidative stress in patients treated with statins. A randomized, crossover clinical study was carried out, with the participation of 53 subjects. At each intervention period, study participants received a packaged for the functional or control treatment. Functional treatment consisted of fish oil (1.7 g of EPA+DHA/day), chocolate containing plant sterols (2.2 g/day) and green tea (two tea sachets/day). Control treatment consisted of soy oil softgels, regular chocolate and anise tea. After 6 weeks of intervention, functional treatment reduced plasma LDL-C (-13.7% ± 3.7, p=0.002) and C-reactive protein (-35.5% ± 5.9, p=0.027). Plasma triacylglycerol (-15.68% ± 5.94, p=0.02) and MDA (-40.98% ± 6.74, p=0.04) were reduced in subgroups of patients (n=23) with baseline values above the median (93 mg/dL and 2.23 umol/L, respectively). Analysis of lathosterol and campesterol in plasma suggested that intensity of LDL-C reduction was influenced by cholesterol absorption rate rather than its endogenous synthesis. After multivariate analysis, patients identified as “good responders” to supplementation (n=10) were recruited for a pilot protocol of statin dose reduction with complementary diet therapy. Responders received the functional treatment for 12 weeks: standard statin therapy was kept during the first 6 weeks and reduced by 50% from weeks 6 to 12. No difference was observed for plasma lipids and inflammation biomarkers, cholesterol efflux capacity or HDL particle number after statin dose reduction when compared to standard therapy. Although limited by the small sample size, our study demonstrates the potential for a new therapeutic approach combining lower statin dose and specific dietary compounds. This may be particularly helpful for the many patients with, and at risk for, CVD who cannot tolerate high-dose statin therapy.

**Keywords:** Dyslipidemia, HDL functionality, statin, omega-3 fatty acids, plant sterols, polyphenols.
ABBREVIATIONS

ABCG5, ATP-binding cassette, sub-family G, member 5;
ABCG8, ATP-binding cassette, sub-family G, member 8;
ACAT2, acetyl-CoA acetyltransferase 2;
ACC, Acetyl-CoA carboxylase;
ALA, α-linolenic acid;
ARA, arachidonic acid;
CAT, catalase;
CE, cholesteryl ester;
EC, cholesterol efflux capacity;
CETP, cholesteryl ester transfer protein;
CM, chylomicron;
CMr, chylomicron remnants;
COX, cyclooxygenase;
CVD, Cardiovascular diseases;
DGAT2, diacylglycerol O-acyltransferase 2;
DHA, docosahexaenoic acid;
EGCG, epigallocatechin-3-gallate;
eNOS, endothelial nitric oxide synthase;
EPA, eicosapentaenoic acid;
FAS, fatty acid synthase;
FC, free cholesterol;
FDA, Food and Drug Administration;
FFA, free fatty acids;
FPS, free plant sterols;
GPx, glutathione peroxidase;
GR, glutathione reductase;
GSH-Px, glutathione peroxidase;
HDL-C, high-density lipoprotein cholesterol;
HDLm, mature high-density lipoprotein;
HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A;
HMG CoA-Reductase, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase;
HOCl, hypochlorous acid;
IDL, Intermediate Density Lipoproteins;
LA, linoleic acid;
LCAT, lecithin-cholesterol acyltransferase;
LDL, low-density lipoprotein (particle);
LDL-C, low-density lipoprotein cholesterol;
LDLr, LDL receptor;
LOX, lipooxygenase;
LPL, lipoprotein lipase;
MAG, monoacylglycerol;
MCP-1, monocyte chemotactic protein 1;
MDA, malondialdehyde;
MM LDL, minimally modified low density lipoprotein;
MPO, myeloperoxidase;
MTP, microsomal triglyceride transfer protein;
n-3 FA, omega-3 fatty acids;
NBT, nitro blue tetrazolium;
NCEP, National Cholesterol Education Program;
NF-kB, nuclear factor kappa B;
NO, nitric oxide;
NOX, NADPH oxidase;
NPC1L1, Niemann-Pick C1 Like 1;
Nrf-2, nuclear factor erythroid 2-related factor-2;
oxLDL, oxidized low density lipoprotein;
PAF, platelet-activating factor;
PCSK9, proprotein convertase subtilisin/keratin type 9;
PHC, phenolic compounds;
PhL, phospholipids;
PMS, phenazinemethosulphate;
PPAR-α, Peroxisome proliferator-activated receptor alfa;
PS, plant sterol;
PSE, plant sterol/stanol esters;
ROS, reactive oxygen species;
SBR1, Scavenger receptor class B member 1;
SOD, superoxide dismutase;
SRA, scavenger receptor class B/A;
SREBP-1c, sterol-regulatory-element-binding protein-1c;
SREBP-2, sterol regulatory element binding protein-2;
TAG, triacylglycerol;
TBARS, thiobarbituric acid reactive substances;
TG, triglycerides;
VCAM, vascular cell adhesion molecule;
VLDL, Very Low Density Lipoproteins;
VLDL-C, Very Low Density Lipoprotein cholesterol.
LIST OF TABLES

Chapter I

Table 1. Strengths and drawbacks of current pharmacological therapies for cardiovascular disease prevention. .................................................................................................................................................. 21
Table 2. Human intervention studies of drug therapy combined to bioactive compounds. ..................... 25
Table 3. Human intervention studies with combination between n-3 fatty acids and plant sterols............. 25
Table 4. Strengths and drawbacks of n-3 FA, PSE, and PHC nonpharmacological therapies for CVD prevention.................................................................................................................................................. 26

Chapter II

Table 1: Subjects characteristics ...................................................................................................................................................................................... 48
Table 2: Anthropometric and biochemical parameters at baseline and after 6 weeks of functional or control treatment ........................................................................................................................................................................... 49
Table 3: Anthropometric and biochemical parameters of Non-Responders (NR) and Responders (R) at the second phase of the study ........................................................................................................................................................................ 51
LIST OF FIGURES

Chapter I

Figure 1: Bioactive compounds with potential cardiovascular protection activity .................................................. 18
Figure 2: Lipid metabolism and the atherosclerotic process. ................................................................................. 19

Chapter II

Figure 1: Baseline sterol profile of subgroups divided according to the intensity of LDL-C reduction after functional treatment.................................................................................................................................................. 53
Figure 2: Spearman correlation graphs for Total Cholesterol Efflux Capacity vs HDL-C, Total HDL particle and CRP, obtained from baseline measures for responders and non-responders .......................................................... 54
Figure S1: Study control markers, before and after each treatment. ............................................................................ 61
Figure S2: Mean changes in plasma EPA+DHA concentration by quartiles according to baseline EPA+DHA values........................................................................................................................................................................... 61
Figure S3: Mean % change of TG and MDA in subgroups of patients according to baseline median ............. 62
Figure S4: Mean % change of LDL and CRP for Responders and Non-Responders after 6 weeks of functional treatment, at the end of first phase of the study. ................................................................. 62
Figure S5: Spearman correlation graphs obtained from baseline measures for Responders and Non-responders........................................................................................................................................................................ 63

Chapter III

Figure 1: HDL isolation technique .......................................................................................................................... 66
Figure 2: Gene expression of M2 markers. .................................................................................................................. 69
Figure 3: M2 polarization experiment.......................................................................................................................... 70
Figure 4: M1 downregulation experiments. .................................................................................................................. 71
Figure 5: Cytotoxicity of cholesterol treatment after 72h. ......................................................................................... 72
Figure 6: Gene expression of SMC marker (α-Actin) and macrophage markers (ABCA1 and CD68) after loading HCASMC with cholesterol (1)......................................................................................................................... 73
Figure 7: Gene expression of SMC marker (α-Actin) and macrophage markers (ABCA1 and CD68) after loading HCASMC with cholesterol (2).......................................................................................................................... 73
Figure 8: Gene expression of SMC marker (α-Actin) and macrophage markers (ABCA1 and CD68) after loading HCASMC with cholesterol (3)..................................................................................................................... 74
Figure 9: Average scan for human HDL particle size distribution............................................................................ 75
Figure 10: Cholesterol efflux capacity of human samples according to treatments ................................................... 76
Figure 11: HDL particle size distribution in human plasma according to treatments ............................................ 77
SUMMARY

EDITORIAL........................................................................................................................................... 14

CHAPTER I. Foods that go (scientifically) perfect together................................................................. 16

Bioactive compounds as an alternative for drug co-therapy: Overcoming challenges in cardiovascular disease prevention ........................................................................................................... 17

Introduction ........................................................................................................................................ 17

Cardiovascular diseases ...................................................................................................................... 18

Pharmacological therapy for cardiovascular disease prevention .......................................................... 20

Bioactive compounds and underlying mechanisms for cardiovascular disease prevention .............. 20

Omega-3 fatty acids ............................................................................................................................... 20

Plant sterol esters .................................................................................................................................. 22

Phenolic compounds .............................................................................................................................. 22

Bioactive compounds as co-therapy with drugs .................................................................................. 23

Considerations and future directions .................................................................................................... 24

Conclusion .......................................................................................................................................... 26

References .......................................................................................................................................... 27

CHAPTER II. “One size will not fit all”............................................................................................ 31

Statin dose reduction with complementary diet therapy: a pilot study of personalized medicine .. 32

Introduction .......................................................................................................................................... 34

Subjects and methods .......................................................................................................................... 35

Results ................................................................................................................................................. 38

Discussion .......................................................................................................................................... 39

Conclusion .......................................................................................................................................... 42

References .......................................................................................................................................... 43

Supporting information .......................................................................................................................... 55
| Section                                                                 | Page |
|------------------------------------------------------------------------|------|
| Supplementary figures                                                 | 61   |
| CHAPTER III. “Can we make HDL great again?”                            | 64   |
| Effects of a combined bioactive supplement on HDL function in statin-treated patients | 65   |
| Introduction                                                           | 65   |
| Optimization of HDL isolation protocol                                 | 66   |
| HDL anti-inflammatory activity                                          | 67   |
| Foam-like Smooth Muscle Cells (SMC) phenotypic reversal                | 72   |
| Cholesterol Efflux capacity (CEC) and HDL particle size in human samples | 74   |
| FINAL CONSIDERATIONS                                                  | 82   |
| ATTACHMENTS                                                           | 83   |
Scientific breakthroughs often rely on advanced and cutting-edge technology. In recent years, the world has witnessed the “Omics” revolution; one major triumph of modern medicine that has been providing massive information about systems biology. All the generated information is comprised of “Big data” sets, that include genomics, transcriptomics, and proteomics, which respectively, describe our genomes, identify which of our genes are being expressed and catalog all the proteins in a tissue sample.

These new and high-throughput molecular analyses provide unprecedented opportunities to revolutionize clinical research and practice, paving the path for precision medicine. Tools as such are essential to expand the understanding of cardiovascular disease (CVD) pathophysiology, find novel targets for drug discovery, and to identify not only patient-specific differences but also particular patient subsets for tailored interventions.

But how much science still must happen to make precision medicine a reality, and shorten the pipeline to interventions that reduce CVD outcomes? Handling and processing these massive data sets is still a big challenge, often overwhelming. Genomic datasets alone have already shown applicability (i.e. gene variants can identify high or low-risk groups for various diseases). However, integration of different types of molecular data remains a bioinformatic/computational challenge, as discoveries are hidden within millions of analyses. Although precision medicine is a much-promised future for disease treatment and prevention, researchers still need to learn how to move from data to knowledge and translate knowledge into action that will ultimately improve the lives of patients in real-world contexts.

Meanwhile, what can be done to improve lives today?

In this present issue, the use of bioactive compounds for CVD prevention is discussed. Bioactive compounds are naturally occurring food components that can beneficially affect target functions in the body, beyond nutritional effects, being especially relevant to the reduction of disease risk. The inclusion of functional foods or bioactive food components as part of one’s diet is a simple and low-cost strategy to improve CVD prevention and patient’s well-being.

Chapter one brings a critical review paper on the potential of bioactive compounds to combat atherosclerosis - the main manifestation of CVD – and how some specific compounds can positively interact with drugs in a synergistic/additive way, or by reaching molecular pathways that single-target drugs cannot reach in a complex disease such as atherosclerosis.
Chapter two describes a clinical study on the metabolic response to a combination of bioactive compounds and a new therapeutic approach of partial replacement of drug dosage by complementary diet therapy – a treatment that could be an alternative for statin-intolerant patients, who may suffer from a reduction of life expectancy or quality of life.

In Chapter three, the prevention of CVD through an emergent and controversial target – High-Density Lipoprotein (HDL) - is briefly discussed, along with potential quantification methods. Final considerations and future directions are also addressed.

References

1. Chance F. The power of big data must be harnessed for medical progress. *Nature*. 2016;539:467-468.
2. Gibbons GH. Charting Our Future Together: Setting an Agenda for the NHLBI. *Circulation*. 2017;136:615-617.
3. Antman EM, Loscalzo J. Precision medicine in cardiology. *Nat Rev Cardiol*. 2016;13:591-602.
4. Winklhofer-Roob BM, Faustmann G, Roob JM. Low-density lipoprotein oxidation biomarkers in human health and disease and effects of bioactive compounds. *Free Radic Biol Med*. 2017;111:38-86.
**CHAPTER I. Foods that go (scientifically) perfect together**

*Research highlights*

Food-drug interactions are often understood as a situation in which the activity of a drug is negatively affected by another substance. Basically, when taken together with medicines, some foods can prevent a drug from acting the way it should, may cause side effects or even worsen a known side effect of a drug. In the following review paper (*CRIT REV FOOD SCI NUTR, 2016*) we discuss the opposite: how can functional foods and bioactive compounds work together with drugs to increase cardiovascular protection?

Major metabolic pathways involved in this protective effect are reviewed and possible interactions with drugs mechanisms of action are pointed out (refer to Figure A1, page 84, for a more complete metabolic view). We go on further and propose that certain ingredients should be taken simultaneously, as they offer complementary benefits. Some foods just go perfectly together to boost your cardiovascular health - although you might have a tough time combining the flavors (you will soon understand).
Bioactive compounds as an alternative for drug co-therapy: Overcoming challenges in cardiovascular disease prevention

Bianca Scolaro, Hellen Soo Jin Kim, and Inar Alves de Castro

Faculty of Pharmaceutical Sciences, Department of Food and Experimental Nutrition, University of São Paulo, São Paulo-SP, Brazil

ABSTRACT

Different pathological mechanisms have been applied with success to reduce the progression of atherosclerosis. However, many patients are not good responders or must interrupt treatment due to adverse effects. Bioactive compounds such as omega-3 fatty acids (n-3 FA), plant sterol esters (PSE) and phenolic compounds (PHC) are natural molecules with great potential to reduce the atherosclerosis burden by reducing inflammation, LDL cholesterol (LDL-C) and oxidative stress, respectively. Although their physiological effects on biomarkers are much lower than those expected by drugs used for the same purpose, bioactive compounds can easily be incorporated into the daily diet and present no adverse effects. However, little is known about the combination of n-3 FA, PSE, PHC, and drugs in atherosclerosis progression. This review article summarizes potential effects of co-therapies involving n-3 FA, PSE, and PHC combined with major hypolipidemic drugs on atherosclerosis biomarkers and clinical outcomes. Evidence of additive and/or complementary effects regarding drugs action reveals possible roles for bioactive compounds in disease management. Pharmaceutical companies, physicians, and food scientists should be prepared to better understand this type of interaction and its consequences in terms of efficacy and life quality.

List of Abbreviations: ALA, a-linolenic acid; OSE, cholesteryl ester; CETP, cholesteryl ester transfer protein; COX, cyclooxygenase; CVD, Cardiovascular diseases; DGA.T2, diacylglycerol O-acyltransferase 2; DHA, docosahexaenoic acid; ECOG, epigallocatechin-3-gallate; enOS, endothelial nitric oxide synthase; EPA, eicosapentaenoic acid; FC, free cholesterol; FDA, Food and Drug Administration; FFA, free fatty acids; FPP, free plant sterol; GSH-Px, glutathione peroxi-dase; HDLC, high-density lipoprotein cholesterol; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LA, linoleic acid; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LDLr, LDL receptor; LOX, lipoxigenase; LPL, lipoprotein lipase; MGP-1, monocyte chemotactic protein 1; MM LDL, minimally modified low density lipo-protein; MPO, myeloperoxidase; n-3 FA, omega-3 fatty acids; NPC1L1, National Cholesterol Education Program; NF-kB, nuclear factor kappa B; NO, nitric oxide; NOX, NADPH oxidase; NPC1L1, Niemann-Pick C1 Like 1; oxLDL, oxidized low density lipoprotein; PAF, platelet-activating factor; PCSK9, Proprotein convertase subtilisin/kexin type 9; PHC, phe-nolic compounds; PPAR-α, Peroxisome proliferator-activated receptor alfa; PSE, plant sterol/stanol esters; ROS, reactive oxygen species; SOD, superoxide dismutase; SREBP-1c, sterol regulatory element-binding protein-1c gene; SREBP-2, sterol regulatory element binding protein-2; TAG, triacylglycerol; VLDLC, very low density lipoprotein cholesterol

Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality in many developed and developing countries (Weber and Noels, 2011; Reiner, 2013; Wong, 2014). Although significant progress has been achieved through new drugs and surgical procedures (Fuster, 2014), the number of deaths caused by CVD is still high. It has been estimated that 23.6 million will die as a consequence of CVD in 2030 (Cannon, 2013). On the basis of 2010 death rate data, one American dies of CVD every 40 seconds. The estimated total cost of this disease is US $315.4 billion, and is projected to rise to US $918 billion in 2030 (Go et al., 2014). For this reason, more efforts have been made in terms of primary prevention, including changes in lifestyle, diet and prescription drugs. However, the effectiveness of these measures is still low due to many factors. For example, it is well-known that the adherence of the patients to chronic drug prescriptions is universally poor, with less than half of those patients who are prescribed antihypertensive or lipid-lowering drugs continuing treatment beyond one year (Chapman et al., 2005). Among the factors that contribute to low patient adherence to medication are the economic burden, intolerance, complexity of treatment, side effects and the number of pills that the patient must take daily (Fuster, 2014; Scicchitano et al., 2014). In a previous study carried out by our group, patients took about eight pills per day, on average (Bertolami et al., 2014). In addition to the adherence limitation, atherosclerosis, the process that underlies CVD, actually starts early in life (Lusis, 2000; Mendis et al., 2005; Rader and Daugherty, 2008) when diet rather than drugs could be used as a preventive intervention. Thus, one alternative that could effectively contribute to early prevention, reduce drug doses or improve the patient response to treatment could be the inclusion of functional foods in the individual’s diet (Eussen et al., 2010).
About two decades ago, food companies started to add bioactive compounds to some food formulations, rendering them “functional.” These bioactive compounds provide specific health benefits when consumed as part of the daily diet. Today, the number of functional foods has increased in different countries, offering several options for consumers. The United States is the world’s largest functional food market with sales up to $43.9 billion in 2012, where six out of ten adults consume functional foods/beverages at least occasionally. Cholesterol-lowering foods/drinks were the most purchased condition-specific food or drink, sought by 29% of consumers (Sloan, 2014).

Despite weaker effects on CVD biomarker improvement when compared with drugs, functional foods do not present any side effects and can be included in the diet from childhood. Patients who are poorly responsive to pharmacological treatments could have a better response if functional foods are viable as a co-therapy, since bioactive compounds can act by different physiological pathways than drugs. In addition, functional foods may contribute to reduce high cholesterol prevalence (Scicchitano et al., 2014). About 5.6% of US adults have undiagnosed hypercholesterolemia and more than half of individuals are at borderline high risk, yet remain unaware of their condition (Go et al., 2014). These individuals could especially benefit from functional foods, since these over-the-counter compounds do not demand a physician’s prescription to be bought in local markets.

Functional foods that are potentially beneficial for CVD prevention include different classes such as fruits, vegetables, legumes, nuts, chocolate, olive oil, fish or fish oil, tea, wine, and soy protein. Dietary patterns and entire diets may also be cardioprotective, particularly the Mediterranean Diet (Alissa and Ferns, 2012). As there is a great number of functional foods and bioactive compounds with large variation of biological activity, this study will focus on main dietary components with anti-inflammatory, cholesterol lowering and antioxidant activity that are readily available for consumption. Among these, omega-3 fatty acids (n-3 FA) from fish and fish oil, plant sterol esters (PSE) found particularly in fortified foods and phenolic compounds (PHC) from green tea and red wine, will be addressed (Figure 1).

The Food and Drug Administration (FDA) in the US recently approved the use of PSE health claim in food labeling, while more evidence is still necessary for n-3 FA and PHC health claims (FDA, 2004; FDA, 2010).

Considering the increase in the functional food market, the combination of these bioactive compounds with drugs is already a reality and will become more evident along with a further reduction in functional foods prices. Thus, it is necessary to understand which biochemical mechanisms are involved when the major hypolipidemic drugs are taken with bioactive compounds. For this reason, the objective of this narrative review was to discuss potential effects of co-therapies involving n-3 FA, PSE and PHC combined with major hypolipidemic drugs on atherosclerosis biomarkers and clinical outcomes. A literature overview was conducted using the following databases: PubMed, Scopus and Web of Science. Articles were identified using the terms: atherosclerosis, statin, niacin, ezetimibe, fibrates, PCSK9 inhibitor, omega-3 fatty acids, plant sterols, phenolic compounds, green tea, wine, resveratrol. Clinical studies on combination therapy, presenting none or positive results as well as adverse effects, were included if they provided useful and clinically relevant information about the efficacy of the treatment and management of dyslipidemia.

### Cardiovascular diseases

Cardiovascular or heart diseases (CVD) include different pathologies that directly affect the heart or vascular system, with high rates of morbidity and mortality (Wong, 2014). The predominant manifestation of CVD is caused by the ischemic heart disease associated with a restriction of the blood flux in arteries, capillaries or veins. In this condition, there is an interruption of the blood supply with severe consequences, such as fatal myocardial infarction (38-46%) or stroke (34-37%) (Wong, 2014).

Atherosclerosis is the process that underlies ischemic diseases and consists of chronic inflammation of the arteries caused by various factors. This condition is often associated with high consumption of lipids rich in saturated and trans fatty acids, cholesterol, simple sugars and salt, sedentarism, overweight or obesity, exposure to an oxidant environment and it is also strongly influenced by hereditary factors (Lusis, 2000). The mechanism proposed to explain atherosclerosis progression in humans involves the infiltration of low-density lipoprotein cholesterol (LDL) particles in the endothelium monolayer intima, where they contribute to monocyte recruitment and to foam cells formation (fatty streaks), as summarized in Figure 2. Briefly, excess LDL infiltrates the first external layer of the endothelium (tunica intima) at sites in the arterial tree where laminar flow is disrupted (Libby, 2002; Rader and Daugherty, 2008). Once retained in the intima, in part binding to proteoglycan, LDL particles are susceptible to oxidative modification by reactive oxygen species (ROS), or by enzymes such as myeloperoxidase (MPO) or lipoxygenase (LOX) released from inflammatory cells (Esterbauer et al., 1992; Weber and Noels, 2011). As the endothelium represents a site of chronic inflammation, ROS within the vessel wall, such as superoxide anions produced by macrophages through the action of membrane-associated NADPH oxidase (NOX) (Heinecke, 1999) will

---

**Figure 1-1:** Bioactive compounds with potential cardiovascular protection activity. Molecule structure of representative omega-3 fatty acids (EPA and DHA), plant sterol/sterolanes (β-sitosterol and β-sitostanol) and phenolic compounds (resveratrol and EGCG). Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EGCG, epigallocatechin-3-gallate.
Figure I-2: Lipid metabolism and the atherosclerotic process.

Cholesterol, plant sterols, phospholipids and triglycerides obtained through diet are incorporated into mixed micelles in the intestinal lumen. Free cholesterol and free plant sterols are absorbed through the NPC1L1 transporter while monoaoylglycerols and diacylglycerols enter into the enterocyte by facilitated diffusion at the brush border. Estenified cholesterol and triacylglycerols are further packed into chylomicrons, which are transported by the lymph to the circulation, delivering free fatty acids to peripheral tissues, through the activity of LPL. Chylomicron remnants undergo hepatic uptake, where contribute to the formation of VLDL along with esterified cholesterol (synthesized through the HMG CoA pathway) and TAG (synthesized through the malonyl-CoA pathway). VLDL particles are secreted into the bloodstream where they deliver FFA to tissues and exchange TAG, CE and PH with mature HDL. Further VLDL hydrolysis gives rise to IDL and LDL particles. LDL distributes CE to tissues and undergoes hepatic uptake through LDL receptors (LDLr). However, LDL particles may infiltrate the endothelial intima where they are retained via matrix proteoglycan binding. The retained LDL particles undergo modification, especially oxidation, resulting in electoreactive LDL and oxidized LDL (oxLDL). This oxidative reaction can also occur in the bloodstream. Oxidized LDL (oxLDL) (see Figure A2, page 85) triggers an inflammatory process that includes monocyte migration, infiltration and differentiation into macrophages. Specific scavenger receptors in the macrophages, such as CD36 and SR-A recognize and internalize oxLDL, leading to the formation of foam cells and fatty streaks. In response to chemoattractants secreted by macrophages and foam cells, smooth muscle cells move into the intima and proliferate, forming a fibrous cap along with extracellular matrix molecules, such as elastin and collagen. With the progression of the atherosclerotic plaque, foam cells undergo apoptosis and give rise to a lipid-rich necrotic core. Proinflammatory mediators, smooth muscle cell death and protease degradation of the extracellular matrix weaken the fibrous cap of the mature plaque, making it susceptible to rupture and inducing the thrombus. The atherosclerotic process may be attenuated by HDL, as it promotes cholesterol efflux from other tissues by scavenger receptor A (SRA) and also from the macrophages. HDL is recognized by SREBP receptors in the liver, keeping the "cholesterol reserve transport" cycle. Drugs and bioactive compounds reduce atherosclerotic risk through the following mechanisms: statins reduce cholesterol synthesis through the inhibition of HMG CoA reductase; PCSK9 antibodies reduce LDL by inhibiting LDLr degradation; ezetimibe and PCSK9 inhibit cholesterol absorption through NPC1L1 competition; and/or also reduces cholesterol from mixed micelles and reduces ACAT2 activity, increasing cholesterol excretion; Niacin reduces TAG hydrolysis in adipose tissue, decreases TAG synthesis through DGAT2 and increases HDL through increased apoA1 synthesis; fibrate also increases HDL through apoA1, increase VLDL hydrolysis through LDL and increase TAG oxidation through PPARα n-3 FA also activates PPARα, thus increasing β-oxidation, and reduces FA synthesis through SREBP-1c downregulation, reduces monocyte infiltration, may reduce endothelial NOX activity and activate eNOS; Phenolic compounds also activate eNOS, reduce the expression of adhesion molecules, reduce the expression of proinflammatory cytokines and diminishes oxidative stress. Abbreviations: ACO3G, ATP-binding cassette, subfamily G, member 5; ACO3G, ATP-binding cassette, subfamily G, member 8; ACAT2, acetyl-CoA acetyltransferase 2; ACC, Acetyl-CoA carboxylase; CE, cholesterol ester; CM, chylomicron; CM, chylomicron remnants; DAG, diacylglycerol; DGAT2, diacylglycerol O-acyltransferase 2; FAS, fatty acid synthase; FC, free cholesterol; FFA, free fatty acids; FFS, free plant sterols; HMG CoA-Reductase, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase; HDL, mature high-density lipoprotein; HOC, hypochlorous acid; IDL, Intermediate Density Lipoproteins; LCAT, lecithin-cholesterol acyltransferase; LDL, lipoprotein lipase; MAL, monoglyceride; MCP-1, monocyte chemotactic protein 1; MPO, myeloperoxidase; MTP, microsomal triglyceride transfer protein; NOX, NADPH oxidases; NPC1L1, Niemann-Pick C1 like 1; oxLDL, oxidized LDL; PCSK9, propionate convertase subtilisin/kexin type 9; PH, phospholipids; PPARα, Peroxisome proliferator-activated receptor alpha; PSE, plant sterol ester; ROS, reactive oxygen species; SRA, scavenger receptor class A; SREBP1c, Scavenger receptor class B member 1; SOO, superoxide dismutase; SREBP1c, sterol regulatory-element-binding protein-1c; SREBP2, sterol regulatory element-binding transcription factor 2; TAG, triacylglycerol; VACM, vascular cell adhesion molecule; VLDL, Very Low Density Lipoproteins.

promote LDL oxidation. In addition, LDL tyrosine residues can also be directly oxidized by hypochlorous acid (HOCl) generated by MPO (Levitan et al., 2010). Oxidized LDL (oxLDL) further induces the release of pro-inflammatory cytokines and monocyte chemotactic protein 1 (MCP-1) (Sanchez-Quesada et al., 2004) and can also inhibit the production of nitric oxide (NO), a chemical mediator with vasorelaxation properties (Lusis, 2000). In the injured endothelium, monocytes transmigrate to the intima, proliferate and differentiate into macrophages (Lusis, 2000). These chemical modifications to LDL induce macrophages to phagocytize the oxLDL recognized by scavenger receptors, such as CD36 and SR-A, leading to foam cell formation (Libby, 2002). In addition, foam cells produce large amounts of MPO, thus driving a vicious cycle (Rose and Afanasieva, 2003). The process also induces vascular smooth muscle cell proliferation in the intima or their migration from
the media layer, causing intimal thickening along with fatty streaks, altering endothelial morphology and narrowing the lumen of the artery (Spagnoli et al., 2007; Rader and Daugherty, 2008). At this point, these cells secrete extracellular matrix proteins such as collagen, increase the aggregation of atherogenic lipoproteins and perpetuate a state of chronic inflammation. The plaque can continue to grow, resulting in clinically obstructive disease known as angina pectoris (Libby, 2002; Rader and Daugherty, 2008). On the other hand, the plaque can undergo abrupt rupture, caused by dissolution of the collagenous matrix of the fibrous cap, exposing the lipid core containing the pro-coagulant protein tissue factor to the vascular lumen, forming a thrombus that suddenly interrupts blood flow, often causing an acute fatal myocardial infarction or stroke (Lusis, 2000; Libby, 2002; Rader and Daugherty, 2008). The inflammatory process not only promotes the initiation and evolution of atheromas, but also contributes to the production of proteolytic enzymes capable of degrading collagen, making the plaque more susceptible to rupture (Libby, 2002). Vulnerable plaques generally have thin fibrous caps and high number of inflammatory cells (Lusis, 2000). Interesting, the atherosclerotic process begins early in life, but generally manifests in older adults (Rader and Daugherty, 2008), when it is too late for preventive interventions, thus limiting the treatment to drugs and surgical procedures, including in this case all negative aspects such as side effects and high costs.

Pharmacological therapy for cardiovascular disease prevention

Current strategies for prevention of cardiovascular events are mainly focused on attenuating hyperlipidemia, while the inflammatory and oxidative mechanisms of atheroprogression are not completely addressed (Weber and Noels, 2011). Although lowering LDL-C is the primary clinical approach to control hyperlipidemia and atherosclerosis risk, the pharmacological management of disease also covers different pathways of lipid metabolism (Figure 2). Strengths and drawbacks of the main drug therapies used to treat and control dyslipidemia are summarized in Table 1.

Bioactive compounds and underlying mechanisms for cardiovascular disease prevention

Omega-3 fatty acids

Polyunsaturated fatty acids (PUFAs) with especially important health effects include those in the omega-6 (n-6) and omega-3 (n-3) fatty acid families. Mammals cannot synthesize α-linolenic acid (ALA, C18:3 n-3) or linoleic acid (LNA, C18:2 n-6), the parent fatty acids of the n-3 and n-6 families, respectively (Adkins and Kelley, 2010; Poudyal et al., 2011). However, humans express enzymes necessary for the conversion of dietary ALA into longer chain PUFAs (LCPUFAs) as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), although these conversions are very limited (De Caterina, 2011). ALA (n-3) and LNA (n-6) share a common metabolic pathway and therefore compete for the first enzyme (Δ6-desaturase) in metabolism, which consequently, represents a limiting step for n-3 LCPUFAs production (Poudyal et al., 2011).

By sharing metabolic pathways, n-3 FA also compete with n-6 FA as substrates for the formation of pro-inflammatory mediators, such as leukotrienes, prostaglandins and cytokines, through complex pathways involving the cyclooxygenase (COX) and LOX enzymes. Altogether, n-6 derived eicosanoids (as PGE2 and LTB4) are pro-inflammatory, while n-3 FA can be enzymatically converted to less active leukotrienes (LTB5) and prostaglandins (PGE3). Thus, displacing the pro-inflammatory n-6 FA pathway reduces the production of pro-inflammatory mediators by substrate competition; this mechanism is thought to be one of the main actions of n-3 FA in reducing inflammation (De Caterina, 2011; Calder, 2012). Furthermore, n-3 FA exert anti-inflammatory and inflammation-resolving roles through other lipid mediators like resolvins and protectins. EPA and DHA give rise to mediators such as resolvins E1 and resolin D1, respectively, while DHA leads to protectin D1. These mediators participate in the resolution of inflammation by limiting its progression and associated damage (Calder, 2012).

Besides reducing inflammation, n-3 FA also reduce the risk of cardiovascular disease associated with dyslipidemia. The hypotriglyceridemic effect of EPA and DHA is well established, and is the result of both increased lipolysis and decreased lipogenesis. These n-3 FA enhance fatty acid β-oxidation via the activation of PPARα. In addition, n-3 FA suppresses transcription of sterol regulatory element-binding protein-1c gene (SREBP-1c), inhibiting de novo lipogenesis by decreasing the expression of some genes like fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (Figure 2). The reduce fatty acid availability for triglycerol (TAG) synthesis indirectly reduces hepatic VLDL synthesis, contributing to the hypotriglyceridemic effect (Nagao and Yanagita, 2008; Tai and Ding, 2010; Mozaffarian and Wu, 2011).

The health effects of EPA and DHA have been highlighted by results from epidemiologic and case-control studies that showed an inverse association between the consumption of fish or fish oil and cardiovascular events or mortality (Daviglus et al., 1997; Albert et al., 1998; Erkikia et al., 2003; Kromhout et al., 2012). After 17 years of follow up, the Physician’s study showed an inverse association between plasma n-3 FA and sudden death from cardiac causes, even among men without a history of cardiovascular disease (Albert et al., 2002). Consistent data were also observed by the Nurse’s Health study, which after 16 years of follow-up showed a lower risk of overall mortality and lower risk of CVD associated with fish and n-3 FA intake, even after results were adjusted for confounding dietary variables (Hu et al., 2002). In the large-scale GISSI Prevenzione trial, treatment with n-3 FA (1 g/day) resulted in significant reductions in all-cause mortality and cardiovascular mortality in patients surviving recent myocardial infarction (GISSI-Prevenzione Investigators, 1999), without affecting the risk of non-fatal coronary events. The authors suggested that the benefit of n-3 FA might not be mediated via antiatherosclerotic and antithrombotic effects, but rather antiarrhythmic, with these effects becoming apparent within only a few months after starting treatment (Marchioli et al., 2002). Thies et al. (2003) showed that treatment with fish oil (1.4 g/day n-3 FA) in patients awaiting carotid endarterectomy promoted the incorporation of n-3 FA in atherosclerotic plaque lipids, and
### Table 1. Strengths and drawbacks of current pharmacological therapies for cardiovascular disease prevention.

| Drug     | Mechanism of action                                                                 | Strengths                                                                                                                                                                                                 | Drawbacks                                                                                                                                                                                                 |
|----------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Statins  | Cholesterol synthesis inhibition, primarily in the liver, by blocking 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the principal rate-limiting enzyme of cholesterol biosynthesis (Braun and Ballantyne, 2011; Lamon-Fava, 2013) | Statins are ubiquitously the first-line drug therapy for LDL-C-lowering. However, they also provide cardiovascular protection beyond their cholesterol-lowering action, called pleiotropic effects, which include anti-inflammatory and antioxidant effects at the vascular wall, thus improving endothelial function and enhancing atherosclerotic plaque stability (Takemoto and Lia, 2001; Ridker et al., 2008; Babeva et al., 2013; Antoniades and Channon, 2014). | Even patients treated with statins have a considerable residual burden of cardiovascular risk (Libby et al., 2011). Additionally, many patients are unable to tolerate the maximal dose of statin therapy, and others do not tolerate any dose at all (Braun and Ballantyne, 2011). Adverse effects range from myalgia to serious muscle damage, which is often accompanied by evidence of renal dysfunction. Cognitive impairment, memory problems and elevation of liver enzymes have also been described (Fernandez et al., 2011; Peasy and Rivera, 2012). |
| Ezetimibe| Cholesterol absorption inhibition, Ezetimibe binds to Niemann-Pick C1 Like 1 (NPC1L1), the principal cholesterol transporter in the brush border of the enterocyte (Reiner et al., 2011). | Ezetimibe is frequently administered in association with statins, particularly in the treatment of homozygous familial hypercholesterolemia or statin intolerant patients. Combined ezetimibe and statin therapy provides an additional 15–20% LDL-C reduction (Reiner et al., 2011). Following nicotinic acid therapy, LDL-C increases in a dose-dependent manner up to 25%, and typically reduces LDL-C by 15–18% and TAG by 20–40% (Remaley et al., 2014). Option treatment for statin-intolerant patients with contraindications for other drugs (like ezetimibe) and who have high LDL-C (Lloyd-Jones, 2014). | Combined therapy with ezetimibe and statins appears to have little effect on the progression of atherosclerosis (Kastelein et al., 2008) and only a modest reduction on the occurrence of cardiovascular events (Cannon et al., 2015) in clinical practice. Main drawback of niacin administration is the occurrence of side effects, especially cutaneous flushing (Olsson, 2010). Even niacin combined with lanopranol (formulation that minimizes facial flushing effects of niacin) was shown to provoke serious adverse events such as an increased occurrence of diabetic complications, serious bleeding, gastrointestinal events, serious infection, myopathy and skin-related events (HPS2-THRIVE study) (Landray et al., 2014). |
| Niacin   | Decreases triacylglycerol (TAG) synthesis by the inhibition of diacylglycerol O-acyltransferase 2 (DGAT2) in the liver, and decreases TAG hydrolysis in adipose tissues and free fatty acid (FFA) flux to the liver. Niacin also increases high-density lipoprotein cholesterol (HDL-C) through the induction of the hepatic production of apoA1 (Chapman et al., 2010; Remaley et al., 2014). | A modest increase in HDL-C (up to 5–15%) can be observed after ezetimibe administration as a consequence of stimulated production of apoA1 and apoAII by PPARα (Ewanga, Enukwude and Wierzbicki, 2013). | Fibrates have been observed to raise plasma creatinine concentration, even though its effects on the kidney are still unknown (Wilkinson et al., 2014). Additionally, fibrates can interact with other drugs. One example is the increased risk of myopathy and rhabdomyolysis resulting from the interaction of gemfibrozil with statins (Chapman et al., 2010; Wilkinson et al., 2014). |
| Fibrates | Fibrate acts as an agonist of peroxisome proliferator-activated receptor alpha (PPARα), which is thought to lower plasma TAG concentrations by activating lipoprotein lipase (LPL) and by decreasing hepatic synthesis of fatty acids (Chapman et al., 2010; Katsuki et al., 2013). | Phase II trials showed that PCSK9 inhibitors, particularly monoclonal antibodies, reduce LDL-C by between 60 and 70%, even in the presence of background statin therapy (Norata et al., 2014; Stein and Paol, 2014). Recently, the FDA approved Praluent (alirocumab) for use in adult patients with heterozygous familial hypercholesterolemia in addition to maximally tolerated statin therapy or patients with clinical atherosclerotic cardiovascular disease such as heart attacks or strokes, who require additional lowering of LDL-C (FDA, 2015; Perkel, 2015). | The lack of conclusive data about possible adverse events and undesirable consequences for the patient, especially that could be related to a very low concentration of LDL-C (Dadu and Ballantyne, 2014), have been some of the obstacles to fully understand these new emerging therapies. For this reason, it is necessary to observe and look forward for more results and data about this new strategy of targeting PCSK9. |

Enhanced atherosclerotic plaque stability, as observed by fibrous cap formation and decreased macrophage infiltration. Years later, the same group conducted another study with similar patients but using n-3 FA as ethyl acids (Omacor), at 1.8 g EPA+DHA/day, rather than the TAG form. The authors once again observed the association between a higher EPA content in the plaque and plaque inflammation and instability (Cawood et al., 2010), which could contribute to lower cardiovascular mortality.

A systematic review that gathered data available until 2012 from randomized controlled trials and clinical trials concluded that n-3 FA intake for at least 6 months reduces cardiovascular events by 10%, cardiac death by 9% and coronary events by 18%, mainly in persons with high cardiovascular risk (Delgado-Lista et al., 2012). Controversially, other large trials have found that n-3 FA supplementation was not associated with major cardiovascular events reduction (Rizos et al., 2012; Roncaglioni et al., 2013). In the Origin trial, for example, consumption of 1 g of n-3 FA acids for six years did not reduce the rate of death from cardiovascular causes or other outcomes in patients at high risk for cardiovascular events (The Origin Trial Investigators, 2012). In the Alpha Omega Trial (Kromhout et al., 2010), low dose of EPA-DHA (400 mg/d) for 40 months did not reduce the rate of cardiovascular events in patients surviving myocardial infarction.
Plant sterol esters

Plant sterols or phytosterols are steroid alcohols synthesized de novo by the isoprenoid pathway, and are responsible for several functions in plant metabolism (Piironen et al., 2000). Plant sterol biosynthesis in higher plants occurs via cycloartenol and also via lanosterol, which is different from yeasts that use only lanosterol (Ohyama et al., 2009). They are derived from squalene and present a molecular structure similar to that of cholesterol (Figure 1). Present as free (FPS) or in an esterified form (PSE), they can contain a double bond in the ring (sterols) or be saturated (stanols) (De Smet et al., 2012). The main plant sterols present in the human diet are β-sitosterol, stigmasterol, campesterol, and brassicasterol, found in vegetable oils, nuts, fruits and cereals (Piironen et al., 2000; Ostlund, 2007).

Due to their relative hydrophobicity, plant sterol absorption involves the cleavage of PSE into FPS in the lumen, solubilization into the emulsified fat phase and formation of a micelle that drives FPS to the brush border membrane (Figure 2), where they are absorbed by the same mechanism as cholesterol, via transporters proteins such as NPC1L1. Once inside the enterocyte cytoplasm, the most part of plant sterols will undergo efflux back to the lumen, mediated by a class of proteins known as ABC transporters, specifically ABCG5 and ABCG8. These two proteins are also expressed on the apical surface of hepatocytes, and are required to export plant sterols into the bile (Davis et al., 2004). The presence of plant sterols in the enterocyte reduces the action of ACAT-2, the enzyme responsible for esterifying sterols, which is a necessary step for their incorporation into chylomicrons by microsomal triglycerides transfer protein (MTP) and further release into the lymph. However, it has been suggested that ACAT-2 reduction may be only a consequence of a reduction in cholesterol flux through the enterocyte (Davis et al., 2004). In addition, during digestion, plant sterols can displace cholesterol in micelle formation, reducing cholesterol absorption. All conjoint mechanisms contribute to a reduced cholesterol (40-60%) and plant sterol (< 15%) absorption rate (De Smet et al., 2012).

Several clinical studies and meta-analyses have shown that consumption of 2-2.5 g/day of PSE promotes a consistent LDL-C reduction of 10%, in average (Miettinen et al., 1995; Ostlund, 2007; Demonty et al., 2009; Ras et al., 2014a; Ras et al., 2014b). Based on epidemiological data and clinical trials with cholesterol-lowering drugs, long-term use of PSE could lower CVD risk up to 20% over a lifetime (Katan et al., 2003). However, as far as we know, no epidemiological study has evaluated the effects of PSE supplementation over CVD outcomes up to now. Available data also suggest a moderate reduction of TAG after PSE intake, with little or no effect over HDL-C and CRP levels (Gylling et al., 2014; Rocha et al., 2016).

In 2010, the FDA approved the health claim for functional foods that provide 1.3 g of plant sterols/day (FDA, 2010). Based on this approval, food companies started to commercialize food products such as margarine, milk, yogurts, biscuits and others containing PSE. In fact, the consumption of these functional foods has been recommended by physicians to patients with hypercholesterolemia, and is nowadays incorporated into National Cholesterol Education Program (NCEP) guidelines (De Smet et al., 2012). A recent study carried out by our group showed that the response in terms of LDL-C reduction varies according to individual differences (Bertolami et al., 2014). This variation depends on an individual’s ability to absorb and synthesize sterols. For example, it has been suggested that individuals with high basal cholesterol synthesis are less responsive to PSE interventions than those who present low endogenous synthesis and, consequently, are better sterol absorbers (Rideout et al., 2010; Mackay et al., 2015). For this reason, measurements of lathosterol and plant sterols in plasma could provide important information about an individual’s capacity for cholesterol synthesis and absorption, respectively, improving the prescription’s efficiency.

Phenolic compounds

Phenolic compounds (PHC) are secondary metabolites in plants and comprise a large class of phytochemicals with different chemical structures (Cheynier, 2012). The phenolic content of plants varies according to the plant and its condition of development (Soto-Vaca et al., 2012), which also influences the type of compound and its corresponding role in the organism. Some examples include the formation of pigments, protection against insects and UV radiation, and antioxidant protection against reactive species (Cheynier, 2012). Evidence from clinical trials and epidemiological studies have suggested that the consumption of fruits and vegetables provides health benefits beyond basic nutrition and also protection against chronic diseases (Dauchet et al., 2009; Boeing et al., 2012), which triggered interest in investigating PHC and their possible role in CVD prevention (Soto-Vaca et al., 2012). Although controversy remains whether PHC-rich food/beverage consumption decreases CVD risk, protective effects of green tea and red wine have been highlighted by numerous studies (Del Rio et al., 2013; Pang et al., 2016).

Green tea

The consumption of green tea predominantly occurs in Asia, especially in countries such as China and Japan (Yang and Wang, 2011; Ozen et al., 2012). Green tea is obtained by processing the leaves of Camellia sinensis, which affects its composition and the amount of phenolic compounds. Processing promotes the inactivation of enzymes and the stabilization of tea components (Yang and Wang, 2011) resulting in 80-90% catechins and 10% other flavanols (Deka and Vita, 2011). Catechins, epigallocatechin-3-gallate (EGCG) (Figure 1), (-)-epigallocatechin (-EGC), (-)-epicatechin-3-gallate (EGC), and (-)-epicatechin (EC) are the main phenolic compounds present in green tea (Yuan et al., 2011).

Epidemiological studies carried out especially in Asia have suggested an inverse association between the risk of cardiovascular disease and elevated consumption of green tea, which could be linked to flavonoids (Moore et al., 2009; Deka and Vita, 2011). Regarding dyslipidemia, an LDL-C lowering effect has been observed after the consumption of flavin-enriched green tea for 12 weeks (Deka and Vita, 2011). Moreover, some studies have suggested beneficial effects of green tea consumption (or extract) in reducing blood pressure, decreasing the risk of diabetes mellitus and diminishing body weight. Even though most of these studies demonstrated a beneficial effect on CVD and metabolic syndrome, some other observational studies did
not obtain the same outcomes, mostly justified by differences of in study characteristics, such as population, dosage, follow-up time and selected biomarkers (Deka and Vita, 2011). Some experimental and human interventional trials have suggested possible mechanisms of action of catechins in the context of reducing CVD events, including anti-inflammatory, anti-proliferative and anti-thrombotic effects (Moore et al., 2009; Deka and Vita, 2011).

Inflammation and endothelial dysfunction play important roles during atherosclerosis development, and catechins have been demonstrated to target some important elements in these process (Naito and Yoshikawa, 2009; Moore et al., 2009; Deka and Vita, 2011). In vitro studies have revealed that EGCG inhibits the migration of neutrophils and macrophages and promotes a reduction in ROS production by inflammatory cells. Regarding endothelial function, EGCG may improve the availability of NO by stimulating eNOS phosphorylation and consequently increasing the production of NO and improving endothelial-dependent vasodilation (Deka and Vita, 2011). In addition, it has been reported that catechins may reduce cellular adhesion molecule expression through the inhibition of ICAM-1/VCAM-1 expression (Naito and Yoshikawa, 2009). Considering the antioxidant effects of green tea, studies have been controversial. However, increased capacity to scavenge ROS has been mentioned as a possible beneficial effect, taking into account the susceptibility of LDL to oxidation (Basu and Lucas, 2007; Deka and Vita, 2011). Despite the fact that there is not yet a clear association between green tea consumption and clinical outcomes, green tea is currently considered a safe drink and a possible healthy choice for CVD risk reduction (Deka and Vita, 2011; Pang et al., 2016).

Red wine

After studies based on the “French Paradox”, which highlighted the association between dietary intake of wine and risk of cardiovascular death, wine has been a target of interest, especially by lowering the prevalence of coronary heart diseases after regular moderate intake (Gresele et al., 2011; Fernandez-Mar et al., 2012). The cardiovascular protection observed after the consumption of red wine and grapes is attributed to its phenolic compound content (Fernandez-Mar et al., 2012). Among them, resveratrol may be the main phenolic related to cardiovascular outcomes (Figure 1) (Szkudelska and Szkudelski, 2010; Gresele et al., 2011). Resveratrol is a stilbene derivative that has been shown to exert beneficial effects as modulation of lipoprotein metabolism, antioxidant activity through the inhibition of ROS (quenching), modulation of platelet function and activation of eNOS (Gresele et al., 2011; Fernandez-Mar et al., 2012). Anti-inflammatory effects have also been reported, including modulation of COX-2 activity (Das and Das, 2010; Tang et al., 2014) and inhibition of phospholipase-D, pro-inflammatory cytokine (IL-1, TNF-alpha, IL-6) and TNF-induced NF-kB activation (Tang et al., 2014).

During initial atherosclerotic lesion formation, resveratrol decreases the expression of adhesion molecules such as ICAM-1/VCAM-1. Moreover, it has been shown to reduce MCP-1 expression through the PI3k/Akt pathway, consequently diminishing monocyte recruitment. Considering foam cells, resveratrol may reduce their formation by modulating cholesterol transport and removal. These effects are related to increased cholesterol efflux, free cholesterol removal, downregulation of oxLDL uptake and stimulation of mature HDL particles (Tang et al., 2014).

A systematic review that gathered results from seven controlled trials concluded that resveratrol supplementation has little effect on lipoprotein metabolism and that cardioprotection may rather be associated with its anti-inflammatory and antioxidant effects (Sahebkar, 2013). Although clinical data about resveratrol is still very limited, many resveratrol supplements are commercially available and widely consumed.

Besides resveratrol, other bioactive compounds, as hydroxytyrosol (Fernandez-Mar et al., 2012) catechins and quercetin (Bertelli and Das, 2009) are present in wine. For this reason, different polyphenols in wine may act together, providing cardiovascular benefits related to wine consumption (Gresele et al., 2011; Calabriso et al., 2016). However, it has been suggested that to attain cardiovascular protection, moderate red wine consumption should focus on wines with high in vitro antioxidant activity (Macedo et al., 2012). Although this kind of information is not available on bottle labels, and health claims for alcoholic beverages are not recommended, the product price may provide an initial indication of its functionality, although this is still under investigation. Llobodanin et al. (2014) evaluated the in vitro antioxidant activity of 666 samples of red wine and concluded that there was an increase in antioxidant activity from US $27.00 to US $37.00/bottle, while above this value no additional benefit in terms of antioxidant activity could be achieved.

More studies are needed to be certain of the beneficial effects of wine in humans, although it is certain that moderate consumption of red wine has an important influence on cardioprotection.

Bioactive compounds as co-therapy with drugs

Some studies have suggested that n-3 FA combined with statin therapy improves lipid biomarkers in hyperlipidemic patients (Barter and Ginsberg, 2008) and may be preferable to other drug combinations (Micalef and Garg, 2009a; Eussen et al., 2010). The Japan EPA Lipid Intervention Study (JELIS) was a pioneering study that evaluated the combination of n-3 FA and statin treatment. The authors observed that EPA supplementation reduced major coronary events by 19% in statin treated patients for secondary prevention, while a non-significant 18% reduction was observed in patients with no history of coronary artery disease (Yokoyama et al., 2007). In the same line of study, the GISSI-HF trial enrolled approximately 7000 patients with heart failure treated with multiagent therapy. The results showed a significant benefit of n-3 FA co-therapy (850–882 mg of EPA plus DHA), which was effective at reducing both all-cause mortality and admissions to hospital for cardiovascular reasons (Tavazzi et al., 2008).

In addition, the COMBOS trial (Davidson et al., 2007) evaluated the lipid lowering efficacy of n-3 FA (4 g/d) combined to simvastatin (40 mg/d) in 254 subjects with persistent hypertriglyceridemia. After eight weeks, the combined treatment significantly reduced non-HDL-C by 9.0%, while the reduction after
placebo plus simvastatin treatment was only 2.2%. The combination of n-3 FA with simvastatin also reduced triglycerides by 29.5%, VLDL-C by 27.5%, significantly increased HDL-C by 3.4% and significantly reduced the total cholesterol:HDL-C ratio (9.6%). Similar results were also observed in the ESPRIT trial (Maki et al., 2013).

Besides plasma lipid lowering effects, n-3 FA in combination to statins may also alter the lipoprotein profile toward less atherogenic particles (Nordoy et al., 2001; Micallef and Garg, 2009a). It was shown that n-3 FA combined with atorvastatin therapy increased the average LDL particle size without increasing the particle concentration (Maki et al., 2011).

Since the combination of n-3 FA with statins seems to be effective, especially on the TAG concentration, adding a third compound to this combination, such as PSE, may provide complementary action on circulating lipids, besides providing more comprehensive health benefits via anti-inflammatory effects and improved vascular function (Micallef and Garg, 2009a). Adding PSE to statin treatment promotes an additive reduction of 10%-15% on total cholesterol and LDL-C, which is similar or even more effective than doubling the statin dose (Katan et al., 2003; Scholle et al., 2009). This mean reduction in LDL-C does not seem to increase significantly further with PSE doses above 2.5 g/day or in long-term consumption. For example, the intake of 3.0 g/day of PSE by type 1 diabetic patients on statin treatment also reduced mean total and LDL-C by 10-16% compared with the baseline values, and by 8-15% compared with the control group (Hallikainen et al., 2011). A mean LDL-C reduction of 10% was also observed after adding even higher doses (5.1 g/d of PSE) to the daily diet of statin treated patients, for eight weeks, in a placebo-controlled clinical trial that enrolled 141 hypercholesterolemic patients (Blair et al., 2000). The authors also observed that this effect was similar for all four types of statins included in the study (atorvastatin, pravastatin, simvastatin, and lovastatin). Furthermore, similar results were observed after long-term consumption of margarine enriched with PSE by statin treated patients (De Jong et al., 2008). Consumption of 2.5 g of plant sterol or stanol esters lowered LDL-C concentrations by 8.7 and 13.1%, respectively, over a period of 1.5 years (De Jong et al., 2008).

There are still few available data about combined therapy with statins and PHC. Naruszewicz et al. (2007) first observed the effects of combined treatment of statins and flavonoids in patients with coronary disease. Supplementation with 255 mg/day of a chokeberry flavonoid extract (about 25% anthocyanins, 50% polymeric procyanidines and 9% phenolic acids), for 6 weeks, significantly reduced oxidative markers as serum 8-isoprostans and the oxLDL concentration (by 38 and 29%, respectively), and also reduced inflammation, as observed by a 23% reduction in hs-CRP (Naruszewicz et al., 2007).

The interaction of moderate consumption of wine and statin treatment has not been studied so far. However, after six months of follow-up, a randomized, placebo-controlled trial evaluated the effects of a resveratrol-enriched grape supplement (Stilvid) in statin-treated patients (Tome-Carneiro et al., 2012). The authors observed that consumption of one capsule of Stilvid daily (containing 350 mg grape polyphenols, including 8 mg resveratrol) significantly decreased apoB (9.8%) and oxLDL (20%) levels, whereas LDL-C was only slightly reduced (4.5%). On the contrary, the consumption of yerba mate (1.7 g of total phenols per day) promoted additional 10.0% and 13.1% reductions in LDL-C after consumption for 20 and 40 days, respectively (p<0.001), in hypercholesterolemic individuals on statin therapy. Polyphenol rich beverage intake also increased HDL-C by 6.2% after 40 days (De Morais et al., 2009). Further improvement in lipoprotein status was also observed after one year of combined treatment with statins and flavonoids (Curtis et al., 2012). Consumption of 27 g/day of a flavonoid-enriched chocolate (containing 850 mg flavan-3-ols [90 mg epicatechin] and 100 mg isoflavones [glycine equivalents]/day) by type 2 diabetic patients significantly reduced LDL-C and the total cholesterol:HDL-C ratio, and attenuated the estimated 10-year risk of CVD (Curtis et al., 2012).

Combination of bioactive compounds and flibe rate has also been investigated. The intake of n-3 FA by hypertriglyceremic patients on flibrate treatment was shown to reduce monocyte secretion of TNF-a, IL-1b, IL-6 and MCP-1, in addition to a significant reduction in TAG (Krysiak et al., 2012). Even patients with severe hypertriglyceridemia stably treated with fenofibrate (130 mg) benefitted from n-3 FA supplementation, showing an additive and significant TAG reduction of 17% (Roth et al., 2009).

There is little or no information regarding treatment with PCSK9 inhibitors and ezetimibe combined with bioactive compounds in humans. Although ezetimibe is a strong pharmacologic inhibitor of intestinal cholesterol absorption, its combination with PSE increases cholesterol fecal excretion and reduces plasma LDL-C, indicating that this compound may act by different pathways on cholesterol metabolism (Lin et al., 2011) (Figure 2).

Regarding to niacin, a pilot study evaluated the effects of n-3 FA and niacin therapy, either alone or in combination. Despite including only a small number of dyslipidemic subjects (n D 29), the results showed that 3.4 g of n-3 FA combined with 3 g of niacin reduced TAG by 50% and increased HDL-C by 30%. The concentration of LDL-C was not altered in either treatment group. Moreover, the addition of n-3 FA did not affect niacin flushing (Isley et al., 2007). A larger study including 60 participants evaluated the effects of extended-release niacin and n-3 FA on metabolic syndrome. The results corroborated previous findings, showing that niacin increased HDL-C, while n-3 FA improved hypertriglyceridemia. Although the LDL-C concentration was not altered by this combination, LDL particle characteristics were changed, leading to less atherogenic forms (Shearer et al., 2012).

Some of the findings on the use of single bioactive compounds as co-therapy with drugs are summarized in Table 2.

**Considerations and future directions**

Altogether, clinical studies regarding bioactive compounds as co-therapy with drugs were mainly designed to investigate the effect of combining statins with n-3 FA or PSE. Among them, trials with n-3 FA enrolled a higher number of patients and for longer periods. The large number of positive results from previous cellular and animal models, added to the wide availability of n-3 FA capsules in the drugstores, was especially responsible for boosting n-3 FA research. On the contrary, combination of
Table 2. Human intervention studies of drug therapy combined to bioactive compounds.

| Combination therapy                                      | Study population | Follow-up | Major findings                                                                 | Ref.          |
|----------------------------------------------------------|------------------|-----------|--------------------------------------------------------------------------------|--------------|
| n-3 FA and statin (1800 mg EPA)                          | All subjects     | 4-6 years | Relative risk reduction of 19% in major coronary events                         | JEGS Yokoyama et al. (2007) |
|                                                           | 9325 EPA         |           |                                                                                |              |
|                                                           | 9319 control     |           |                                                                                |              |
|                                                           | 8897 EPA         |           |                                                                                |              |
|                                                           | 6900 control     |           |                                                                                |              |
| n-3 FA and multiagent therapy (850–882 mg of EPA plus DHA) | 6975 subjects with heart failure | 3-9 years | Reduction of all-cause mortality and admissions to hospital for cardiovascular reasons | GISSHIF Tavazzi et al. (2008) |
| n-3 FA and statin (4 g/d FA)                             | 254 subjects on stable statin treatment | 8 weeks  | 9% non-HDL cholesterol reduction; 29.5% TAG reduction; 27.5% VLDL reduction and 3.4% HDL increase | COMDOS Davidson et al. (2007) |
| n-3 FA and statin (4 g/d FA)                             | 432 patients with persistent hypertriglyceridemia | 6 weeks  | 6.9% non-HDL cholesterol reduction; 20.6% TAG reduction | ESPRIT Maki et al. (2013) |
| n-3 FA and statin (4 g/d FA)                             | 237 subjects with mixed dyslipidemia | 8 weeks  | Increase in average LDL particle size without particle concentration increase | Maki et al. (2011) |
| n-3 FA and statin (400 mg of EPA plus DHA)               | 4153 patients with history of myocardial infarction | n-3 FA co-therapy had no effect on major cardiovascular events, despite a significant reduction in triglycerides | Essen et al. (2012) |
| n-3 FA and PSE (3 g/d PSE)                               | 24 type 1 diabetic patients | 4 weeks  | LDL reduction of 10-16% compared to baseline, and Hallikainen et al. (2011) | Blair et al. (2000) |
| n-3 FA and PSE (5.1 g/d PSE)                             | 141 hypercholesterolemic patients | 8 weeks  | LDL incremental reduction of 10%, regardless statin type | (2011)       |
| n-3 FA and PSE (2.5 g/d PSE)                             | 54 subjects on stable statin treatment | 1.5 years | LDL reduction of 8.7–13.1% | De Jong et al. (2008) |
| Flavonoids and statin (255 mg/d chokeberry extract)      | 44 patients with history of myocardial infarction | 6 weeks  | 38% reduction of 8-isoprostanes; 29% reduction of oxLDL and 23% reduction of hsCRP | Naruszewicz et al. (2007) |
| Grape supplement and statin (8 mg/d 75 subjects on stable statin treatment resveratrol) | 102 subjects | 60 days | Reduction of 9.8% in ApoB and 20% in oxLDL | Tome-Caimeiro et al. (2012) |
| Yerba mate and statin (1.7 g of total phenols)           | 93 type 2 diabetic patients | 1 year   | Additional 13.1% reductions in LDL and 6.2% increase in HDL | Curtis et al. (2012) |
| Flavonoid enriched chocolate and statin (850 mg flavan-3-ols and 100 mg isoflavones) | 46 subjects with isolated hypertriglyceridemia | 90 days  | Significant reduction on LDL and attenuated the estimated 10-year risk of CVD | De Morais et al. (2009) |
| n-3 FA and fibers (4 g/d FA)                             | 58 patients with severe hypertriglyceridemia stably treated with fenofibrate | 8 weeks  | 35% reduction in TAG and reduction on monocyte secretion of TNFalpha, IL-1 beta, IL-6 and MCP-1 | Kyslaik et al. (2012) |
| n-3 FA and niacin (3.4 g/d FA)                            | 29 dyslipidemic subjects | 12 weeks | TAG reduction of 50% and HDL increase of 30%. | Isley et al. (2007) |
| n-3 FA and niacin (4 g/d FA)                             | 60 metabolic syndrome subjects | 16 weeks | TAG reduction of 34% and HDL increase by 7.8 moli/dl; LDL particles changed to the less atherogenic, large, buoyant particles | Shearer et al. (2012) |
| Abbreviations: n-3 FA, omega 3 fatty acids; TAG, triacylglycerol; PSE, plant sterol esters.                  |                  |           |                                                                                |              |

Table 3. Human intervention studies with combination between n-3 fatty acids and plant sterols.

| Combination dosage                                      | Study population | Follow-up | Main findings                                                                 | Ref.          |
|----------------------------------------------------------|------------------|-----------|--------------------------------------------------------------------------------|--------------|
| phytosterol-enriched spread (2 g/d) and n-3 FA capsules  | 60 hyperlipidemic subjects | 3 weeks  | LDL reduction of 12.5%, HDL-C increase of 8.6% and TAG reduction of 25.9% hsCRP reduction of 39% | McAllef and Garg (2008; 2009b) |
| spreads containing a fixed amount of PSE (2.5 g/ day) and varying amounts of EPACDA (0.0, 0.9, 1.3 and 1.8 g/day) | 314 hypercholesterolemic subjects | 4 weeks  | LDL-C reduction of 13% and TAG reduction of 9–16% in dependence on the EPACDA dose | Ras et al. et al. (2014); Jacobs et al. (2015) |
| spreads containing a fixed amount of PSE (2.5 g/ day) and varying amounts of EPACDA (0.0, 0.9, 1.3 and 1.8 g/day) | 282 hypercholesterolemic subjects | 4 weeks  | Shifts in the lipoprotein distribution: VLDL particles were reduced in cholesterol and TAG content; HDL particles were increased in cholesterol and TAG | Ras et al. et al. (2014); Jacobs et al. (2015) |
| 2 g/d plant sterol (yogurt drink) and 2 g/d omega-3 fatty acids from fish oil (capsules) | 178 mildly hypercholesterolemic patients (total cholesterol 5.0–8.0 mmol/l) | 4 weeks  | LDL-C reduction 4.2% and non-HDL-C reduction of 3.9% (non-significant) | Khandelwal et al. (2013) |
effect after intervention, although differences among biomarkers and clinical outcomes were observed. While some compounds act on the same biochemical parameter as drugs, complementary effects by reduction of a secondary target can also be observed after co-therapy. Lack of effect has also been reported, and can be attributed to the fact that statin treatment may dilute the preventive cardiovascular effects of bioactive compounds, as it has been previous suggested for n-3 FA (Eussen et al., 2012). Nevertheless, studies so far have not associated any risk to the different combinations that have been tested.

Diet therapy may not only contribute to increase responsiveness to drugs but also to increase treatment compliance. One of the proposed strategies to increase treatment adherence, and that has been gaining strength in the last years, is the use of “Polypill”—a pill that combines four to five drugs (Wiley and Fuster, 2014). However, the Polypill concept of fixed-dose combination goes against the current trend of personalized medicine. Rather than multidrug combination, we believe that future efforts should focus on multi-bioactive combination. A few studies already observed the effects of combining n-3 FA and PSE (Table 3). In a recent study carried out by our group, hyperlipidemic patients were supplemented with a combination of n-3 FA, PSE and green tea (data not shown). We observed a reduction on lipid parameters (LDL-C and TAG), as well as inflammatory and oxidative stress markers. However, the results were strongly related to patient’s individual characteristics. In general, the outcomes observed from these studies suggest both synergistic and complementary effects through multi-bioactive combination.

Considering the results observed for drug combination with bioactive compounds and for multi-bioactive combination, we believe that all mechanistic pathways attributed to each individual compound/drug (that are summarized in Table 1 and in Figure 2) can be simultaneously activated during treatments and do not interfere with each other. This is especially relevant in terms of dietary co-therapy and highlights the potential for new food products development where different compounds may be present in the same food matrix. Current strengths and drawbacks for the use n-3 FA, PSE and PHC in CVD prevention are summarized in Table 4.

### Conclusion

The combination of bioactive compounds with drugs appears to be a safe and effective therapy to reduce CVD progression. However, many drug interactions with bioactives, especially phenolic compounds, are still poorly understood and documented. Although data so far indicate a potential additive and/or complementary effect, large clinical trials are still necessary to evaluate changes in biomarkers and clinical outcomes. Based on this information, physicians may recommend specific combinations to improve patient’s treatment. It is worthy to highlight that patients respond individually to bioactive compounds, as well as to drugs. Thus, the effect of bioactive compounds and drug combination will be particular to each patient. The number of bioactive compounds sold in the drugstores for individuals who aim to prevent or treat CVD shows that the combination is already a reality. Pharmaceutical companies, physicians and food scientists should be prepared to better understand this type of interaction and its consequences in terms of efficacy and life quality.

### Competing interests

The authors declare that they have no competing interests.

### Funding

This study was financially supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP; Process 14/04247-3 and 15/16243-5) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 163211/2013-2). The funders had no role in decision to publish, or preparation of the manuscript.

### Authors’ contributions

BS designed the manuscript; BS, IAC and HSKJ wrote the paper; IAC had primary responsibility for final content. All authors read and approved the final manuscript.
Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: A randomised controlled trial. Lancet. 361:477–485.
Tome-Carneiro, J., González, M., Larrosa, M., García-Almagro, F. J., Avíles-Plaza, F., Parra, S., Yáñez-Gascon, M. J., Ruiz-Ros, J. A., García-Conesa, M. T., Tomas-Barberan, F. A. and Espín, J. C. (2012). Consumption of a grape extract supplement containing resveratrol decreases oxidized LDL and ApoB in patients undergoing primary prevention of cardiovascular disease: A triple-blind, 6-month follow-up, placebo-controlled, randomized trial. Mol. Nutr. Food Res. 56:810–821.
Weber, C. and Noels, H. (2011). Atherosclerosis: Current pathogenesis and therapeutic options. Nat. Med. 17:1410–1422.
Wiley, B. and Fuster, V. (2014). The concept of the polypill in the prevention of cardiovascular disease. Ann. Glob. Heal. 80:24–34.
Wilkinson, M. J., Laffin, L. J. and Davidson, M. H. (2014). Overcoming toxicity and side-effects of lipid-lowering therapies. Best Pract. Res. Clin. Endocrinol. Metab. 28:439–452.
Wong, N. D. (2014). Epidemiological studies of CHD and the evolution of preventive cardiology. Nat. Rev. Cardiol. 11:276–289.
Yang, C. S., and Wang, H. (2011). Mechanistic issues concerning cancer prevention by tea catechins. Mol. Nutr. Food Res. 55:819–831.
Yokoyama, M., Origasa, H., Matsuzaki, M., Matsuzawa, Y., Saito, Y., Ishi-kawa, Y., Oikawa, S., Sasaki, J., Hishida, H., Itakura, H., et al. (2007). Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded end-point analysis. Lancet. 369:1090–1098.
Yuan, J. -M., Sun, C. and Butler, L. M. (2011). Tea and cancer prevention: Epidemiological studies. Pharmacol. Res. 64:123–135.
Research highlights

Atherosclerosis is an extremely complex disease. No wonder why one drug alone cannot fully prevent cardiovascular events (like myocardial infarction) from happening. On top of that, many patients develop side effects from statins – the main group of drugs for cholesterol lowering. Muscle pain, which often can be debilitating, drives patients to stop taking the pills, leaving them even more vulnerable.

In the following manuscript\(^1\), we first demonstrate that statin-treated patients taking all three bioactive compounds - omega-3 fatty acids, plant sterols, and polyphenols – show an improvement of atherosclerosis risk factors, such as cholesterol and inflammation. We continue to show that if statin dose is reduced by half while taking these bioactive compounds, the levels of cholesterol and other biomarkers are kept stable. Thus, patients suffering from side effects, especially from high-intensity statin therapy, could have their dose reduced by introducing these compounds in the diet, at specific amounts. However, not all patients may benefit from this new therapy, as “one size does not fit all”.

\(^1\) Submitted to BMC Medicine.
STATIN DOSE REDUCTION WITH COMPLEMENTARY DIET THERAPY: A PILOT STUDY OF PERSONALIZED MEDICINE

Bianca Scolaro\textsuperscript{a}, Marina Nogueira\textsuperscript{a}, Aline Paiva\textsuperscript{a}, Adriana Bertolami\textsuperscript{b}, Lucia P. Barroso\textsuperscript{c}, Tomas Vaisar\textsuperscript{d}, Sean Heffron\textsuperscript{e}, Edward Fisher\textsuperscript{e}, Inar Castro\textsuperscript{a*}

\textsuperscript{a} Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Lineu Prestes, 580, B14 - 05508-900, São Paulo, Brazil
\textsuperscript{b} Dyslipidemia Medical Section, Dante Pazzanese Institute of Cardiology, Av. Dr. Dante Pazzanese, 500, 04012-909 São Paulo, Brazil
\textsuperscript{c} Department of Statistics, Institute of Mathematics and Statistics, Rua do Matão, 1010, 05508-090, São Paulo, Brazil.
\textsuperscript{d} Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195, USA
\textsuperscript{e} Leon H. Charney Division of Cardiology, Department of Medicine, New York University School of Medicine, New York, NY, 10016, USA

*Address for correspondence: *Inar Alves Castro: LADAF (www.ladaf.com.br). Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Lineu Prestes, 580, B14 - 05508-900 São Paulo, Brazil phone number: +55 11 3091 1481 e-mail: inar@usp.br

Short running title: Statin dose reduction for responders to dietary compounds
ABSTRACT

Background: Statin intolerance, whether real or perceived, is a growing issue in clinical practice. Complementary diet therapy can contribute to enhance primary cardiovascular disease prevention by reducing adverse effects and number of add-on prescriptions.

Objective: Our aim was to evaluate the effects of reduced-dose statin therapy complemented with a combined supplementation of omega-3 fatty acids, plant sterols and polyphenols, compared to standard statin therapy.

Methods: First phase: a randomized crossover study was carried out on 53 type 2 diabetic patients treated with statins. Patients received a functional treatment that consisted of fish oil (1.7 g of EPA+DHA/day), chocolate containing plant sterols (2.2 g/day) and green tea (two tea sachets/day). Second phase: after multivariate analysis, patients identified as responders to supplementation (n=10) were recruited for the pilot protocol. Patients received the functional treatment for 12 weeks. Standard statin therapy was kept during the first 6 weeks and reduced by 50% from weeks 6 to 12.

Results: First phase: functional treatment reduced plasma LDL-C (-13.7±3.7, p=0.002) and C-reactive protein (-35.5±5.9, p=0.027). Analysis of lathosterol and campesterol in plasma suggested that intensity of LDL-C reduction was influenced by cholesterol absorption rate rather than its endogenous synthesis. Second phase: no difference was observed for plasma lipids and inflammation biomarkers, cholesterol efflux capacity or HDL particle number after statin dose reduction when compared to standard therapy.

Conclusions: Although limited by the small sample size, our study demonstrates the potential for a new therapeutic approach combining lower statin dose and specific dietary compounds. Further studies should elucidate “good responders” profile as a tool for personalized medicine. This may be particularly helpful in the many patients with, and at risk for, CVD who cannot tolerate high dose statin therapy.

Trial registration: ClinicalTrials.gov, NCT02732223. Retrospectively registered on April 4, 2016.

Keywords: Atherosclerosis, omega-3 fatty acids, plant sterols, polyphenols, responders
INTRODUCTION

Cardiovascular disease (CVD), particularly atherosclerosis, is the major cause of morbidity and mortality throughout the world. The main target for CVD prevention is LDL-cholesterol (LDL-C) lowering, usually attained by statins [1]. Although statins are considered a first-line lipid-lowering therapy and have expressively contributed to clinical event reduction, residual cardiovascular risk remains high among statin-treated patients [2]. The most recent study with PCSK9 antibodies (FOURIER trial) supported the LDL-C hypothesis which is “the lower the better”, showing that this new class of drugs can further decrease the rate of clinical events [3]. However, results have also shown that despite extreme LDL-C reduction, absolute outcomes (MI) were still high after PCSK9 inhibition atop statin therapy [3]. Furthermore, atherosclerotic plaque regression with PCSK9 antibody was previously shown to be only 1% [4] highlighting the need to address risk factors beyond lipids, such as inflammation and oxidative stress. This approach is especially important under diabetic conditions, when plaque regression is impaired in pre-clinical models even when LDL-C is normalized [5].

Novel add-on therapies to maximally tolerated statin have also focused on the atheroprotective properties of HDL, especially its function on reverse cholesterol transport. However, the effectiveness of HDL-targeted agents is still controversial [6]. Although combination therapies have proven necessary [7,8] and despite increasing efforts in drug development, a critical issue that undermines CVD prevention is the high prevalence of statin undertreatment due to real or perceived adverse effects [9]. Statin intolerance leads to discontinuation or suboptimal statin use; afflicted patients have 50% higher risk of coronary events than those with good statin adherence [10]. Convincing these patients, that claim muscle pain or weakness, to continue the statin therapy is a major challenge in clinical practice.

The combination of nutraceuticals with statin treatment could help overcome these limitations [11,12]. Bioactive compounds such as omega-3 fatty acids (n-3 FA), plant sterols and polyphenols are naturally occurring molecules with great potential to reduce atherosclerosis progression by reducing inflammation, LDL-C and oxidative stress, respectively [13–16]. Although the biological effects of these single compounds have been extensively evaluated over the last decade [15,17,18], studies regarding bioactive compound combinations are still scarce, and the combined action of such agents in atherosclerosis is poorly understood [19]. More interestingly, the potential of reducing statin dose in conjunction with complementary diet therapy has never been investigated, and could contribute to reduce adverse effects associated to high statin dosages and increase treatment adherence.
In the present study, we first evaluated the effects of daily consumption of a combined supplementation of n-3 FA (fish oil supplement), plant sterols (enriched chocolate) and polyphenols (green tea) on biomarkers of inflammation, lipidemia and oxidative stress in type 2 diabetic patients treated with statins. We then conducted a pilot study to evaluate - in terms of plasma lipid profile, inflammatory and oxidative markers - the effects of statin dose reduction by the combined supplementation compared to standard statin therapy (full dose), in a sub-group of patients who better responded to the initial intervention.

SUBJECTS AND METHODS

Subjects
Participants with established dyslipidemia and diabetes were recruited from Dante Pazzanese Institute of Cardiology (São Paulo, Brazil). The primary selection criteria were current statin (simvastatin or atorvastatin) and hypoglycemic treatment (metformin and/or gliclazide). Exclusion criteria were patients who were taking n-3 FA, plant sterol or green tea supplements; poorly controlled diabetes (HbA1c > 7.5%) or dyslipidemia (LDL-C > 100 mg/dL); pregnant females; and patients who presented any congenital cardiac disorders, uncontrolled endocrine, renal or hepatic disease and excessive alcohol consumption. Based on these criteria, 53 subjects (male n = 19 and female n = 34) were enrolled and completed the first phase of the trial. Patient characteristics are shown in Table 1.

Study design
First phase: a randomized, placebo-controlled, crossover intervention was carried out. The protocol was single-blinded once the quantification of ingestion markers in plasma samples was indicative of subject’s treatment. Initially, subjects were randomly assigned to receive functional or control treatment for 6 weeks. Daily functional treatment (FNT) consisted of seven fish oil softgels (1.7 g of EPA+DHA), two dark chocolate truffles containing plant sterol esters (2.2 g/day) and two green tea sachets (~170.8 mg epigallocatechin gallate (EGCG)/day). Control treatment (CON) consisted of seven soy bean oil softgels, two regular dark chocolate truffles and two anise tea sachets. As green tea and anise tea taste different, participants were informed in the beginning of the protocol that the tea taste would change along the study. The chocolate containing plant sterols was developed with collaboration of Chocolife Indústria e Comércio de Alimentos Funcionais Ltda, São Paulo [20]. The plant sterols PinVita™ES (70% β-sitosterol) from DuPont™ Danisco® Food Ingredients were purchased from MasterSense Ing. Alim. Ltda. (São Paulo, Brazil). The fish oil and placebo supplements (1 g softgels) were donated by Bionatus Laboratório Botânico Ltda. (São José do Rio
Preto, São Paulo). The green tea and anise tea sachets (1.6 and 2.0 g each) were donated by Leão Alimentos e Bebidas (São Paulo, Brazil). Detailed description of chemical composition and oxidative status of food components are available in Appendix S1 (Supporting Information).

Participants were instructed to consume 1 softgel after breakfast, 3 softgels and 1 chocolate truffle twice a day (after main meals), and to drink two cups of tea per day. After a 6-week washout period the groups were changed following the cross-over design for 6 weeks more. During each step, the subjects maintained their habitual routine and diet. Prescribed drugs were kept without any change throughout the study.

Second phase: Multivariate statistical tools were applied to identify subjects that overall better responded to the combined supplementation provided in the first phase of the trial. Cluster analysis was performed including C-reactive protein, malondialdehyde and LDL-C concentration as active variables. Via this analysis, two sub-groups were identified with the aim of classifying patients that showed greater and lesser degrees of responses to the functional intervention. Subjects that made up the so-called responder (“R”; n=10) and non-responder (“NR”; n=10) sub-groups were invited to participate in the second phase of the study. A pilot protocol of statin dose reduction by complementary diet therapy was carried out in the “R” sub-group only. Initially, “R” and “NR” subjects were recruited for a first visit, when baseline blood samples were collected and the functional treatment (FNT) was provided only to responders. The “R” sub-group completed 6 weeks of FNT during which they maintained their prescribed dose of statin. Responders then reduced their statin dose 50%, and completed 6 more weeks of functional treatment.

Anthropometric data and biochemical measures
Anthropometrical measures (weight, waist circumference, abdominal circumference and hip circumference) were recorded at each visit. All blood samples were collected at baseline and after each intervention. Blood was drawn in vacutainer tubes (EDTA for plasma and SST for serum) after at least a 12 h fast. Plasma and serum were separated by centrifugation at 1000 × g for 5 min at 4 °C (Hitachi, CF-15R, Tokyo, Japan). Total cholesterol, HDL-C, LDL-C, triglycerides, glucose, creatine kinase, urea, alanine transaminase (ALT), aspartate transaminase (AST), glycated haemoglobin (Hb A1c) and high sensitivity C-Reactive Protein (hs-CRP) were performed utilizing commercial kits (Labtest Diagnostica SA, Lagoa Santa, Brazil) with automated system Labmax 240 (Labtest Diagnostica SA, Lagoa Santa, Brazil). Plasma EPA, DHA and arachidonic acid (ARA, n-6) were measured using gas chromatography (GC-MS) as described by Shirai et al. (2005) [21]. Quantification of main plant sterols (campesterol and β-sitosterol) and cholesterol precursor
(lathosterol) in plasma was performed according to García-Llatas et al. (2012) using gas chromatography (GC-MS) [22]. Plasma malondialdehyde (MDA) concentration was assessed using reverse phase HPLC [23]. Activity of the antioxidant enzymes superoxide dismutase (SOD) [24], catalase (CAT) [25], glutathione peroxidase (GPx) [26] and glutathione reductase (GR) [27] was determined in erythrocyte lysates. Cholesterol efflux capacity (CEC) of apolipoprotein-B depleted plasma samples was determined in vitro as total, ABCA1-specific and non-ABCA1-specific efflux of radiolabeled cholesterol from macrophages to the plasma, as described [28]. HDL particle size was determined by ion mobility analysis (IMA) [29]. A detailed description of methodologies is available in Appendix S1 (Supporting Information).

**Statistical analysis**

Values are expressed as mean ± standard error of mean (SEM). Variance homogeneity and normality were evaluated for all variables. **First phase:** Percent changes from baseline after each treatment (CTR and FNT) were compared with independent samples T-tests. Data were also compared by dividing the patients into subgroups: patients who presented baseline values above the median and patients who presented baseline values below the median for each biomarker. In these comparisons, the percent of change observed in each subgroup was compared between the two treatments with independent samples T-tests. **Second phase:** A Principal Component Analysis was applied to separate the patients according to their responses in terms of plasma lipid profile, inflammatory and oxidative stress biomarkers. The first and second components explained 75.38% of the variation. On the basis of the projection of the cases on the factor-plane, 20 patients were randomly recruited from opposite directions, and classified as “Responders” and “Non-Responders” to the functional treatment. Differences between these two groups were compared by Mann-Whitney U tests. Data obtained only for “Responders” submitted to both treatments (standard statin therapy vs half dose statin) were compared with Wilcoxon-tests. In this last comparison, error type II is also important, since the objective of the study was to confirm the null hypothesis. For this reason, the β risk was evaluated estimating a change of 6% for all variables as clinically relevant. The β risk was calculated based on normal distribution and Lehmann method using the software G*Power (Allgemeine Psychologie und Arbeitspsychologie, Dusseldorf, Germany). Spearman correlations were used to assess associations between biomarkers separately within the responder and non-responder groups. Significance was set at p <0.05. Analyses were performed with the software STATISTICA version 9.0 (StatSoft, Inc., Tulsa, OK, USA).
RESULTS

Functional treatment effect on atherosclerosis risk biomarkers

Plasma changes of adherence markers are shown in Figure S1. Mean omega-3/omega-6 ratio (EPA+DHA/ARA) and β-sitosterol/cholesterol ratio increased after functional treatment, indicative of subject compliance. As expected, increases in EPA+DHA concentration after functional treatment were higher for individuals who presented with lower baseline values (Figure S2).

The changes in anthropometric and biochemical parameters of individuals according to the type of intervention are shown in Table 2. Baseline values of all parameters were equal for both treatments, suggesting that the 6-week washout period in our crossover design was sufficient to return the biomarkers to their respective original values. The functional treatment was effective in reducing total cholesterol (-10.1%), LDL-C (-13.7%), non-HDL-C (-12.0%) and hs-CRP (-35.5%). No changes were observed for the other parameters. However, functional treatment reduced TG (-17%) and MDA (-31%) in a subgroup of patients with baseline values above the median (93 mg/dL and 2.23 umol/L, respectively) (Figure S3). When individuals were grouped according to the intensity of LDL-C reduction after the functional treatment (Figure 1), it was observed that higher LDL-C reduction was independent of baseline lathosterol values (p=0.988) - a sterol synthesis marker – but was dependent on sterol absorption, represented by campesterol concentration at baseline (p=0.045), suggesting that individuals characterized as a “sterol absorber” rather than “sterol synthesizer” would respond to plant sterol supplementation with a greater LDL-C reduction.

Pilot study of statin dose reduction

Results obtained in the first phase of the study showed that combined intake of n-3 FA, plant sterols and polyphenols reduced lipemia and markers of inflammation and oxidative stress in some Type 2 diabetic, statin-treated patients. Based on these results, a cluster analysis was performed aiming to group patients with greater and lesser degrees of responses to the functional intervention. Ten patients from each cluster were invited to participate in the second phase. Figure S4 shows the percent change of LDL and hs-CRP for “R (Responders)” and “NR (Non-Responders)”, measured at the end of the first phase. No difference was observed in MDA between the groups (p=0.910). At the beginning of the second phase (pilot study), “R” and “NR” groups characteristics were compared (Table 3). We observed that “R” exhibited higher concentration of HDL-C (p=0.031) and lower small HDL particle concentration (p=0.045) than “NR”. Although not significant, “R”
showed a trend toward higher concentration of large HDL particles (p=0.063) and non-ABCA1 specific cholesterol efflux capacity (p=0.075) than “NR”.

A positive linear correlation was observed between total CEC and HDL-C (r=0.825; p=0.003) and total HDL particle (r=0.847; p=0.002) for “R” (Figure 2). As to “NR”, a less strong correlation was observed between total CEC and total HDL particle (r=0.620; p=0.056), while it was positively correlated to VLDL-C (r=0.769; p=0.009) and negatively correlated with hs-CRP (r=-0.781; p=0.008) (Figures 2 and S5). ABCA1-specific CEC was also negatively correlated with hs-CRP for “NR” (r=-0.821; p=0.004). TG and VLDL-C were positively correlated with ABCA1-specific CEC for both “R” (r=0.662; p=0.037 and r=0.791; p=0.006) and “NR” (r=0.882; p=0.001 and r=0.861; p=0.001) (Figure S5).

Regarding the pilot study, standard statin therapy (SST) was compared with half dose statin therapy complemented with a functional intervention (HDS). No differences were observed between both treatments for all biomarkers evaluated in this study (Table 3), and β risk was lower than 5% just for parameters associated with body weight (data not shown), suggesting that for some patients, functional intervention can effectively combat any change in biomarkers despite a reduction in statin dose.

DISCUSSION

Several epidemiological and clinical studies have investigated the effect of isolated bioactive compounds on biomarkers of atherosclerosis and end-points involved with cardiovascular diseases [15,17,18]. This is the first clinical study that investigated the effect of a concomitant combination of plant sterols, n-3 FA and polyphenols on dyslipidemia, inflammation and oxidative stress markers in patients treated with statins.

Our results showed that the supplementation was easily incorporated into daily routine, as observed by plasma concentrations of EPA+DHA and β-sitosterol after 6 weeks of functional treatment. Despite high adherence, the biochemical response to the combined bioactive supplementation varied according to the biomarkers. It was observed that TG reduction was relative to TG baseline values, being significant only in the subgroup of patients with baseline values above the median. The hypotriglyceridemic effect of n-3 FA is well established; increased β-oxidation, via activation of PPARα, and decreased lipogenesis by suppression of sterol regulatory element-binding protein-1c (SREBP-1c) gene transcription are the described mechanisms [30]. Previous data show that the magnitude of n-3 FA’s hypotriglyceridemic effect is both influenced by baseline TG concentration and n-3 FA dose, suggesting that clinically significant TG reduction occurs only if EPA+DHA dose exceeds 2 g/day [13]. In our study, despite an estimated daily intake of 2.1 g/d of
EPA+DHA, quantification of fatty acids in softgels showed that the provided EPA+DHA dose was 1.7 g/d. In a recent study, Kleiner and colleagues showed that over 70% of commercial n-3 FA supplements sold in the United States did not contain the stated label amount of EPA and DHA [31], partially due to oxidative instability [32]. Interestingly, human clinical trials usually do not report the oxidative status of supplements, which may be in part responsible for conflicting results [33]. More rigorous quality control should be applied to commercial n-3 FA capsules, as consumption of oxidized lipids can be deleterious [34]. Nevertheless, TG reduction achieved by fish oil supplements seems to be beneficial in patients at risk of CVD, since hypertriglyceridemia is strongly associated with atherosclerosis pathogeneses and may also contribute to residual cardiovascular risk [35].

Plant sterol consumption decreased LDL-C by 13%, independent of LDL-C baseline values, which corroborates previous studies [36,37] and may correspond to a reduction of 20% in CVD risk over a lifetime [18]. The intensity of LDL-C reduction was shown to be influenced by subject’s cholesterol absorption rate (as judged by basal campesterol concentration). Controversially, previous reports showed that basal lathosterol measures (cholesterol synthesis marker) could predict an individual’s LDL-C lowering response to plant sterol supplementation, as lathosterol concentration negatively correlated with intensity of LDL-C reduction after intake of plant sterols [38,39]. However, these observations were conducted with untreated hypercholesterolemic individuals, while subjects in our study were treated with statins and therefore had limited lathosterol synthesis.

A limitation in our study was that statin treatment was not restricted to a single type (atorvastatin or simvastatin) and dose. However, we suggest that treatment with different statins did not affect the LDL-C response to plant sterols, since baseline cholesterol synthesis was similar between subgroups with lowest and highest LDL-C reduction. The same was reported by Hallikainen and colleagues [40], when individuals using three different statins with varying doses were provided with plant stanol esters. These authors observed that neither the type, nor the dose, of statin had influence over the results.

As stated above, the degree of LDL-C reduction was associated with the baseline cholesterol absorption rate. Whether statin treatment was responsible for upregulating cholesterol absorption rate remains to be determined [41]. This could be better elucidated if lathosterol and campesterol measurements were taken in all patients prior to statin therapy assignment. In fact, identifying an individual’s sterol synthesizer/absorber profile could be a powerful tool for personalized medicine in primary prevention. The information about sterol synthesizer/absorber profile would allow clinicians to introduce cholesterol absorption inhibitors, like ezetimibe or plant sterols, at the beginning of treatment, preventing adverse effects of high statin doses.
In our study, functional treatment also reduced hs-CRP, possibly through an additive anti-inflammatory effect of n-3 FA and polyphenols. N-3 FA, especially EPA and DHA, give rise to inflammation-resolving mediators like resolvins and protectins [42]. Increased availability of n-3 FA also reduces synthesis of pro-inflammatory eicosanoids (through substrate competition with omega-6 fatty acids) and decreases activation of NF-kB [42,43]. However, n-3 FA effects on CVD risk remains inconclusive [44]. The addition of n-3 FA to statin therapy was previously shown to reduce hs-CRP by 22% after 4 g EPA/day [45]. In our study, 1.7g/day EPA+DHA prompted a 35% hs-CRP reduction. Likewise, the consumption of a polyphenol extract by statin-treated patients was previously shown to reduce hs-CRP by 23%, accompanied by a 29% reduction of oxidized LDL [46]. In the present study, a reduction of MDA (lipid oxidation secondary product) was observed only in a subgroup of patients with higher baseline values, possibly promoted by the antioxidant activity of green tea polyphenols, since both groups received the same amount of chocolate. Although green tea intake has been widely associated with antioxidant and anti-atherosclerotic effects [47], results have been inconsistent and there is a lack of high-quality studies linking green tea intake to CVD risk reduction [48].

A critical principle of personalized medicine is to provide the right therapy to the right patient at the right time [49]. Our study shows for the first time that, for some patients, statin dosage can be reduced by half, without change in biomarkers of CVD risk, provided that specific dietary compounds are consumed daily at pre-specified amounts. This is consistent with previous observations that doubling statin dose causes only about 6% additional decrease of LDL-C [50], which can easily be attained by plant sterol supplementation. The effect of statin therapy on HDL metabolism is less clear. Previous reports have shown that statins may both decrease [51,52] or increase [53] CEC - the only metric of HDL functionality that has been shown to be predictive of prospective CVD events. A recent study showed that on-statin CEC was inversely associated with incident cardiovascular events and that, in fact, HDL particle number was the strongest HDL-related biomarker of residual risk in statin-treated patients [54]. In our pilot study, a 50% reduction on statin dosage did not alter CEC or HDL particle concentration.

The individual characteristics that distinguish “R” from “NR” subjects remain to be elucidated. Despite having 40% more HDL-C, responders did not show marked alteration in CEC, compared to non-responders. Although both groups also had similar HDL particle concentrations, we observed a shift in size distribution, with “R” having fewer small particles and higher concentrations of large particles, which carry most of the HDL cholesterol. “R” and “NR” subjects may have a different inflammatory context since smaller HDL particles have been associated with anti-inflammatory properties [55] and hs-CRP was negatively correlated with CEC for non-
responders. Further studies are necessary to better understand and predict response to both drugs and nutraceuticals.

Apart from our small sample size, another limitation of our study is that we did not compare the effects of reduced statin dose by itself, without any supplementation. Therefore, it is not clear if the 6 weeks of reduced-dose statin therapy was adequate to induce statistical changes in biomarkers. However, it would not be prudent or advisable to lower statin dose of high-risk patients without providing an alternative treatment. Long-term studies are necessary to confirm that biomarkers are kept unchanged during HDS therapy.

CONCLUSION

The combined intake of n-3 FA, plant sterols and polyphenols promoted changes thought to be cardioprotective when added to statin therapy, observed by reductions of lipids, and of markers of inflammation and oxidative stress. Furthermore, it became clear that the hypocholesterolemic effect depends on the individual’s sterol synthesis/absorption capacity. Although limited by the small sample size, our study has also shown the potential for adding nutraceuticals to statin therapy for primary prevention, instead of for example, doubling drug dosage whenever LDL-C levels are suboptimal. This may be particularly helpful in the many patients with, and at risk for, CVD who cannot tolerate high-dose statin therapy. Such approach however, should be particular to subjects identified as good responders to the proposed treatment. Further studies are needed to establish new markers as a mean to predict individual response to nutraceuticals.

ABBREVIATIONS

ARA, arachidonic acid; CAT, catalase; CEC, cholesterol efflux capacity; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EGCG, epigallocatechin gallate; EPA, eicosapentanoic acid; GPx, glutathione peroxidase; GR, glutathione reductase; MDA, malondialdehyde; n-3 FA, omega-3 fatty acids; PPARα, peroxisome proliferator-activated receptor alpha; SOD, superoxide dismutase; SREBP-1c, sterol regulatory element-binding protein-1c; TG, triglycerides.

DECLARATIONS

ACKNOWLEDGEMENTS

We are grateful to the staff of the Hospital, especially Dr. Andre Faludi, Chief of the Dyslipidemia Medical Section of Dante Pazzanese Institute of Cardiology. We also thank Dr. Marcelo Bertolami who provided insight and expertise that greatly assisted the research.
Funding
This work was financially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo- FAPESP (Process 14/04247-3, 14/18576-9, 15/16243-5, 15/18859-3) and National Institutes of Health grant P01HL092969.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
BS, IC and AB designed the study. BS, IC, AP and AB conducted clinical aspects of the study. BS, MN, AP and TV conducted laboratory aspects of the study. BS, IC, LPB, TV, SH and EF analyzed and interpreted the data. Critical revision of the manuscript for important intellectual content: IC, SH and EF. BS wrote first draft of the manuscript and has primary responsibility for final content. All authors contributed to subsequent drafts, and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of the Dante Pazzanese Institute of Cardiology (CAAE 27349114.5.3001.0067) and all patients provided informed written consent prior to inclusion.

REFERENCES

1. Gotto AM, Moon JE. Pharmacotherapies for lipid modification: beyond the statins. Nat. Rev. Cardiol. 2013;10:560–70.
2. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.
3. Sabatine MS, Giugliano RP, Keech AC, Honarpour N, Wiviott SD, Murphy SA, et al. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. N. Engl. J. Med.
4. Nicholls SJ, Puri R, Anderson T, Ballantyne CM, Cho L, Kastelein JJP, et al. Effect of Evolocumab on Progression of Coronary Disease in Statin-Treated Patients. JAMA. 2016;316:2373.

5. Parathath S, Grauer L, Huang L-S, Sanson M, Distel E, Goldberg JJ, et al. Diabetes Adversely Affects Macrophages During Atherosclerotic Plaque Regression in Mice. Diabetes. 2011;60:1759–69.

6. Kingwell Ba, Chapman MJ, Kontush A, Miller NE. HDL-targeted therapies: progress, failures and future. Nat. Rev. Drug Discov. 2014;13:445–64.

7. Chapman MJ, Redfern JS, McGovern ME, Giral P. Niacin and fibrates in atherogenic dyslipidemia: pharmacotherapy to reduce cardiovascular risk. Pharmacol. Ther. 2010;126:314–45.

8. Davidson MH. Reducing residual risk for patients on statin therapy: the potential role of combination therapy. Am. J. Cardiol. 2005;96:3K–13K.

9. Stoekenbroek RM, Kastelein JJP. Dyslipidaemia: Statin-associated muscle symptoms — really all in the mind? Nat. Rev. Cardiol. 2017;14:445–6.

10. Serban M, Colantonio LD, Manthripragada AD, Monda KL, Bittner VA, Banach M, et al. Statin Intolerance and Risk of Coronary Heart Events and All-Cause Mortality Following Myocardial Infarction. J. Am. Coll. Cardiol. 2017;69:1386–95.

11. Rideout TC, Harding S V, Marinangeli CPF, Jones PJH. Combination drug-diet therapies for dyslipidemia. Transl. Res. 2010;155:220–7.

12. Moss JWE, Ramji DP. Nutraceutical therapies for atherosclerosis. Nat. Rev. Cardiol. 2016;13:513–32.

13. Jacobson TA. Role of n-3 fatty acids in the treatment of hypertriglyceridemia and cardiovascular disease. Am. J. Clin. Nutr. 2008;87:1981S–1990S.

14. De Smet E, Mensink RP, Plat J. Effects of plant sterols and stanols on intestinal cholesterol metabolism: suggested mechanisms from past to present. Mol. Nutr. Food Res. 2012;56:1058–72.

15. Manach C, Mazur A, Scalbert A. Polyphenols and prevention of cardiovascular diseases. Curr. Opin. Lipidol. 2005;16:77–84.

16. Scolaro B, Soo Jin Kim H, de Castro IA. Bioactive compounds as an alternative for drug co-therapy: Overcoming challenges in cardiovascular disease prevention. Crit. Rev. Food Sci. Nutr. 2016;8398:1–14.

17. Lee JH, O’Keefe JH, Lavie CJ, Harris WS. Omega-3 fatty acids: cardiovascular benefits, sources and sustainability. Nat. Rev. Cardiol. 2009;6:753–8.

18. Katan MB, Grundy SM, Jones P, Law M, Miettinen T, Paoletti R. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin. proceedings.
19. Moss JWE, Davies TS, Garaiova I, Plummer SF, Michael DR, Ramji DP. A Unique Combination of Nutritionally Active Ingredients Can Prevent Several Key Processes Associated with Atherosclerosis In Vitro. PLoS One. 2016;11:e0151057.

20. Botelho PB, Galasso M, Dias V, Mandrioli M, Lobato LP, Rodriguez-Estrada MT, et al. Oxidative stability of functional phytosterol-enriched dark chocolate. LWT - Food Sci. Technol. 2014;55:444–51.

21. Shirai N, Suzuki H, Wada S. Direct methylation from mouse plasma and from liver and brain homogenates. Anal. Biochem. 2005;343:48–53.

22. García-Llatas G, Vidal C, Cilla A, Barberá R, Lagarda MJ. Simultaneous quantification of serum phytosterols and cholesterol precursors using a simple gas chromatographic method. Eur. J. Lipid Sci. Technol. 2012;114:520–6.

23. Hong Y, Yeh S, Chang C, Hu M. Total Plasma Malondialdehyde Levels in 16 Taiwanese College Students Determined by Various Thiobarbituric Acid Tests and an Improved High-Performance Liquid Chromatography-based Method. Clin. Biochem. 2000;33:619–25.

24. Ewing JF, Janero DR. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anal. Biochem. 1995;232:243–8.

25. Nabavi SF, Habtemariam S, Sureda A, Hajizadeh Moghaddam A, Daglia M, Nabavi SM. In vivo protective effects of gallic acid isolated from Peltiphyllum peltatum against sodium fluoride-induced oxidative stress in rat erythrocytes. Mol Cell Biochem. 2013;372:233–9.

26. Flohé L, Günzler WA. Assays of glutathione peroxidase. Methods Enzymol. 1984;105:114–20.

27. Torres LL, Quaglio NB, de Souza GT, Garcia RT, Dati LMM, Moreira WL, et al. Peripheral oxidative stress biomarkers in mild cognitive impairment and Alzheimer’s disease. J. Alzheimer’s Dis. 2011;26:59–68.

28. De La Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. Arterioscler. Thromb. Vasc. Biol. 2010;30:796–801.

29. Hutchins PM, Ronsein GE, Monette JS, Pamir N, Wimberger J, He Y, et al. Quantification of HDL Particle Concentration by Calibrated Ion Mobility Analysis. Clin. Chem. 2014;60:1393–401.

30. Mozaffarian D, Wu JHY. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. J. Am. Coll. Cardiol. 2011;58:2047–67.

31. Kleiner AC, Cladis DP, Santerre CR. A comparison of actual versus stated label amounts of EPA and DHA in commercial omega-3 dietary supplements in the United States. J. Sci. Food Agric. 2015;95:1260–7.
32. Albert BB, Derraik JGB, Cameron-Smith D, Hofman PL, Tumanov S, Villas-Boas SG, et al. Fish oil supplements in New Zealand are highly oxidised and do not meet label content of n-3 PUFA. Sci. Rep. 2015;5:7928.
33. Albert BB, Cameron-Smith D, Hofman PL, Cutfield WS. Oxidation of marine omega-3 supplements and human health. Biomed Res. Int. 2013;2013:464921.
34. Nogueira MS, Kessuane MC, Lobo Ladd AAB, Lobo Ladd F V., Cogliati B, Castro IA. Effect of long-term ingestion of weakly oxidised flaxseed oil on biomarkers of oxidative stress in LDL-receptor knockout mice. Br. J. Nutr. 2016;116:258–69.
35. Ng TWK, Ooi EMM, Watts GF, Chan DC, Barrett PHR. Atorvastatin plus omega-3 fatty acid ethyl ester decreases very-low-density lipoprotein triglyceride production in insulin resistant obese men. Diabetes, Obes. Metab. 2014;16:519–26.
36. Scholle JM, Baker WL, Talati R, Coleman CI. The effect of adding plant sterols or stanols to statin therapy in hypercholesterolemic patients: systematic review and meta-analysis. J. Am. Coll. Nutr. 2009;28:517–24.
37. Bertolami A, Botelho PB, Macedo LFL, Abdalla DSP, Faludi AA, Galasso M, et al. Effect of plant sterols compared with ezetimibe on oxidative stress in patients treated with statins. J. Funct. Foods. 2014;10:178–86.
38. Rideout TC, Harding S V, Mackay D, Abumweis SS, Jones PJH. High basal fractional cholesterol synthesis is associated with nonresponse of plasma LDL cholesterol to plant sterol therapy. Am. J. Clin. Nutr. 2010;92:41–6.
39. Mackay DS, Gebauer SK, Eck PK, Baer DJ, Jones PJH. Lathosterol-to-cholesterol ratio in serum predicts cholesterol-lowering response to plant sterol consumption in a dual-center, randomized, single-blind placebo-controlled trial. Am. J. Clin. Nutr. 2015;101:432–9.
40. Hallikainen M, Kurl S, Laakso M, Miettinen TA, Gylling H. Plant stanol esters lower LDL cholesterol level in statin-treated subjects with type 1 diabetes by interfering the absorption and synthesis of cholesterol. Atherosclerosis. 2011;217:473–8.
41. Van Himbergen TM, Matthan NR, Resteghini NA, Otokozawa S, Ai M, Stein EA, et al. Comparison of the effects of maximal dose atorvastatin and rosuvastatin therapy on cholesterol synthesis and absorption markers. J. Lipid Res. 2009;50:730–9.
42. Calder PC. The role of marine omega-3 (n-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability. Mol. Nutr. Food Res. 2012;56:1073–80.
43. Li K, Huang T, Zheng J, Wu K, Li D. Effect of marine-derived n-3 polyunsaturated fatty acids on C-reactive protein, interleukin 6 and tumor necrosis factor α: a meta-analysis. PLoS One. 2014;9:e88103.
44. Rangel-Huerta OD, Aguilera CM, Mesa MD, Gil A. Omega-3 long-chain polyunsaturated fatty
acids supplementation on inflammatory biomarkers: a systematic review of randomised clinical trials. Br. J. Nutr. 2012;107:S159–70.

45. Ballantyne CM, Bays HE, Kastelein JJ, Stein E, Isacsohn JL, Braeckman R a., et al. Efficacy and safety of eicosapentaenoic acid ethyl ester (AMR101) therapy in statin-treated patients with persistent high triglycerides (from the ANCHOR study). Am. J. Cardiol. 2012;110:984–92.

46. Naruszewicz M, Łaniewska I, Millo B, Dłuzniewski M. Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infarction (MI). Atherosclerosis. 2007;194:179–84.

47. Pang J, Zhang Z, Zheng T, Bassig BA, Ge J, Yang Y, et al. Green tea consumption and the risk of the related factors of cardiovascular diseases and ischemic related diseases: A meta-analysis. Int. J. Cardiol. 2016;202:967–74.

48. Murray M, Walchuk C, Suh M, Jones PJ. Green tea catechins and cardiovascular disease risk factors: Should a health claim be made by the United States Food and Drug Administration? Trends Food Sci. Technol. 2015;41:188–97.

49. Ridker PM. How common is residual inflammatory risk? Circ. Res. 2017;120:617–9.

50. Reiner Z. Combined therapy in the treatment of dyslipidemia. Fundam. Clin. Pharmacol. 2010;24:19–28.

51. Ronsein GE, Hutchins PM, Isquith D, Vaisar T, Zhao XQ, Heinecke JW. Niacin Therapy Increases High-Density Lipoprotein Particles and Total Cholesterol Efflux Capacity but Not ABCA1-Specific Cholesterol Efflux in Statin-Treated Subjects. Arterioscler. Thromb. Vasc. Biol. 2016;36:404–11.

52. Nicholls SJ, Ruotolo G, Brewer HB, Kane JP, Wang MD, Krueger KA, et al. Cholesterol efflux capacity and pre-beta-1 HDL concentrations are increased in dyslipidemic patients treated with evacetrapib. J. Am. Coll. Cardiol. 2015;66:2201–10.

53. Kini AS, Vengrenyuk Y, Shameer K, Maehara A, Purushothaman M, Yoshimura T, et al. Intracoronary Imaging, Cholesterol Efflux, and Transcriptomes After Intensive Statin Treatment. J. Am. Coll. Cardiol. 2017;69:628–40.

54. Khera A V., Demler O V., Adelman SJ, Collins HL, Glynn RJ, Ridker PM, et al. Cholesterol Efflux Capacity, High-Density Lipoprotein Particle Number, and Incident Cardiovascular EventsClinical Perspective. Circulation. 2017;135:2494–504.

55. Duprez DA, Otvos J, Tracy RP, Feingold KR, Greenland P, Gross MD, et al. High-Density Lipoprotein Subclasses and Noncardiovascular, Noncancer Chronic Inflammatory-Related Events Versus Cardiovascular Events: The Multi-Ethnic Study of Atherosclerosis. J. Am. Heart Assoc. 2015;4:e002295.
Table II 1: Subjects characteristics

|                        | n   | Mean ± SEM     |
|------------------------|-----|----------------|
| Gender (M/F), n        | 19/34 | -              |
| Ethnicity (White; Black; Asian) | 42;10;1 | -              |
| Age (y)                | -    | 63.8±0.9       |
| Systolic blood pressure (mm Hg) | -    | 130.7±2.9     |
| Diastolic blood pressure (mm Hg) | -    | 80.2±1.7      |
| Heart rate (bpm)       | -    | 69.9±1.7       |
| Medical follow up (y)  | -    | 10.4±0.9       |

Drugs

| Drug                              | n   |                  |
|-----------------------------------|-----|-----------------|
| Atorvastatin (20;40;80 mg)        | 5;12;6 | -               |
| Sinvastatin (10;20;40 mg)         | 1;16;13 | -              |
| Metformin 500 – 2550 mg (1 – 3 daily) | 46*  | -               |
| Gliclazide 30 – 120 (1 – 3 daily)  | 12*  | -               |
| Aspirin (100 mg)                  | 39 | -               |
| Diuretic                          | 42  | -               |
| Angiotensin converting enzyme (ACE) inhibitor | 14  | -               |
| Angiotensin II receptor antagonist | 26  | -               |
| Beta-blocker                      | 18  | -               |
| Calcium channel blocker           | 15  | -               |
| Alpha2 adrenergic agonist         | 2   | -               |
| Vasodilator                       | 2   | -               |
| Antiocoagulant                    | 2   | -               |
| Antiarrhythmic agent              | 2   | -               |
| Thyroid hormone thyroxine         | 7   | -               |
| Hyporuricemic agent               | 6   | -               |
| Proton pump inhibitor             | 13  | -               |

* 5 subjects were on treatment with both metformin and glicazide
Table II 2: Anthropometric and biochemical parameters at baseline and after 6 weeks of functional or control treatment

| Parameter                        | Control treatment (CTR) | Functional treatment (FNT) | p1   | p2   |
|----------------------------------|-------------------------|----------------------------|------|------|
|                                  | Baseline                | After 6 weeks              | % change | Baseline | After 6 weeks | % change |       |       |
| Weight (kg)                      | 85.9±3.7                | 86.4±3.8                   | 0.4±0.2 | 85.6±3.7 | 85.7±3.6 | 0.2±0.3 | 0.948 | 0.616 |
| BMI (kg/m²)                      | 32.4±1.1                | 32.6±1.1                   | 0.4±0.2 | 32.2±1.0 | 32.3±1.0 | 0.2±0.3 | 0.936 | 0.616 |
| Waist circumference (cm)         | 100.6±2.5               | 100.4±2.6                  | -0.3±0.4 | 100.8±2.4 | 100.1±2.3 | -0.5±0.4 | 0.955 | 0.733 |
| Abdominal circumference (cm)     | 104.3±2.7               | 104.4±2.6                  | 0.1±0.3 | 105.7±2.5 | 104.4±2.4 | -1.0±0.6 | 0.710 | 0.076 |
| Hip circumference (cm)           | 111.4±2.3               | 111.2±2.3                  | -0.1±0.4 | 111.1±2.3 | 111.6±2.3 | 0.4±0.4 | 0.946 | 0.271 |
| Glucose (mg/dL)                  | 102.1±4.7               | 108.0±4.7                  | 11.4±5.4 | 100.3±3.4 | 104.7±3.7 | 7.26±3.8 | 0.776 | 0.532 |
| HbA1c (%)                        | 6.5±0.1                 | 6.6±0.1                    | 1.7±1.2 | 6.5±0.1 | 6.4±0.1 | -0.6±1.2 | 0.910 | 0.181 |
| Total cholesterol (mg/dL)        | 170.9±6.5               | 171.8±5.3                  | 5.5±4.0 | 171.9±5.9 | 151.5±5.7 | -10.1±2.7 | 0.913 | 0.002 |
| LDL-C (mg/dL)                    | 86.1±4.6                | 84.9±3.5                   | 7.9±5.7 | 90.1±4.8 | 74.1±3.9 | -13.7±3.7 | 0.543 | 0.002 |
| Non-HDL-C (mg/dL)                | 113.3±5.3               | 116.4±3.4                  | 10.3±4.8 | 115.4±5.0 | 98.3±4.6 | -12.0±3.4 | 0.776 | 0.000 |
| HDL-C (mg/dL)                    | 54.8±1.9                | 53.0±1.9                   | -0.75±3.6 | 53.7±1.6 | 50.7±1.5 | -3.6±2.7 | 0.634 | 0.527 |
| VLDL-C (mg/dL)                   | 29.7±2.4                | 33.2±1.8                   | 24.9±6.0 | 28.3±2.6 | 25.0±1.7 | 5.3±8.1 | 0.704 | 0.054 |
| TG (mg/dL)                       | 146.1±13.4              | 150.1±12.6                 | 10.7±6.2 | 129.8±14.6 | 125.2±7.8 | 16.7±8.5 | 0.413 | 0.564 |
| CPK                              | 152.4±15.7              | 129.1±13.3                 | -10.4±5.9 | 158.0±16.7 | 139.4±14.0 | -0.1±6.8 | 0.807 | 0.254 |
| AST (U/L)                        | 28.2±1.7                | 27±1.2                     | 4.22±3.4 | 30.8±1.4 | 31.0±1.7 | 2.1±3.8 | 0.240 | 0.678 |
| ALT (U/L)                        | 23.9±2.0                | 24.3±1.6                   | 8.5±7.7 | 26.3±1.6 | 28±2.2 | 7.9±5.0 | 0.356 | 0.946 |
Values are expressed as mean ± SEM (n=53). Percent changes from baseline were calculated for each treatment. Functional treatment consisted of a combined supplementation of fish oil softgels, plant sterol enriched chocolate truffles and green tea. Control treatment consisted of soy bean oil softgels, regular dark chocolate truffles and anise tea sachets. p1: probability value obtained by independent samples T-test for baseline values between functional and control treatment. p2: probability value obtained by independent samples T-test between % change after functional and control treatments.

|                     | Control treatment |                      | Functional treatment |                      | p1   | p2   |
|---------------------|-------------------|----------------------|----------------------|----------------------|------|------|
|                     | Baseline          | After 6 weeks        | % change             | Baseline             | After 6 weeks | % change |
| hs-CRP (mg/L)       | 2.1±0.3           | 1.4±0.21             | -10.9±9.3            | 2.1±0.3              | 1.2±0.2   | -35.5±5.9 |
| MDA (umol/L)        | 2.4±0.2           | 1.7±0.1              | -1.2±11.8            | 2.2±0.1              | 1.8±0.1   | -1.7±11.1 |
| Glutathione peroxidase (U/mL) | 9.5±0.3 | 9.2±0.3              | 3.9±6.1              | 8.5±0.3              | 8.1±0.2   | 1.7±4.18  |
| Glutathione reductase (U/mL) | 0.081±0.003 | 0.084±0.003          | 10.44±5.53           | 0.082±0.004          | 0.083±0.003 | 6.52±4.32 |
| Superoxide dismutase (U/mL) | 9.6±0.2 | 8.2±0.3              | -12.3±3.6            | 9.4±0.2              | 8.2±0.2   | -10.6±3.4 |
| Catalase (U/mL)     | 56.9±1.5          | 63.2±2.2             | 14.9±5.1             | 57.0±1.6             | 70.0±2.4  | 26.1±5.2  |

(Table II 2 continued)
|                          | NR (n=10) baseline | R (n=10) baseline/SST | R HDS | p1   | p2   |
|--------------------------|-------------------|----------------------|-------|------|------|
| Age (y)                  | 62.7±2.0          | 62.1±2.4             | -     | -    | -    |
| Gender (M/F), n          | 4/6               | 3/7                  | -     | -    | -    |
| Atorvastatin (20;40;80 mg)| 1;2;0             | 1;1;1                | -     | -    | -    |
| Sinvastatin (20;40)      | 3;4               | 4;3                  | -     | -    | -    |
| Weight (kg)              | 86.0±11.0         | 75.9±5.6             | 76.9±5.8 | 0.526 | 0.939 |
| BMI (kg/m²)              | 32.0±2.5          | 29.5±1.8             | 29.9±1.8 | 0.526 | 0.879 |
| Waist circumference (cm) | 99.7±6.6          | 91.9±4.3             | 93.0±4.3 | 0.407 | 0.879 |
| Abdominal circumference (cm) | 106.3±5.6        | 98.9±3.6             | 98.6±3.5 | 0.661 | 0.650 |
| Hip circumference (cm)   | 114.0±5.8         | 104.8±3.4            | 106.0±3.5 | 0.306 | 0.677 |
| Glucose (mg/dL)          | 115.8±11.9        | 121.5±17.1           | 121.6±6.5 | 0.910 | 0.174 |
| Total cholesterol (mg/dL) | 178.4±9.9        | 210.7±19.6           | 196.3±12.7 | 0.241 | 0.597 |
| LDL-C (mg/dL)            | 101.9±8.7         | 117.0±14.7           | 109.0±9.5 | 0.762 | 0.791 |
| HDL-C (mg/dL)            | 43.4±3.2          | 61.3±6.1             | 62.6±5.6 | **0.031** | 0.939 |
| VLDL-C (mg/dL)           | 33.1±4.9          | 32.4±6.6             | 24.7±2.0 | 0.850 | 0.427 |
| TG (mg/dL)               | 215.8±45.2        | 165.3±50.2           | 114.7±14.6 | 0.162 | 0.623 |
| hs-CRP (mg/L)            | 2.2±0.4           | 3.4±0.91             | 2.6±0.8 | 0.521 | 0.791 |
| Total HDL particle (µM)  | 19.5±0.8          | 22.6±1.4             | 23.0±1.8 | 0.104 | 0.850 |
| Extra Small HDL particle (µM) | 1.4±0.1        | 1.4±0.1              | 1.5±0.2 | 0.910 | 0.969 |
| Small HDL particle (µM)  | 6.6±0.8           | 4.7±0.8              | 4.4±0.7 | **0.045** | 0.733 |
|                                | NR (n=10) baseline | R (n=10) baseline/SST | R HDS | p1    | p2    |
|--------------------------------|-------------------|-----------------------|-------|-------|-------|
| Total small (xs+s) HDL particle (µM) | 7.9±0.7           | 6.2±0.7               | 5.9±0.7 | 0.064 | 0.623 |
| Medium HDL particle (µM)         | 8.5±0.6           | 10.6±1.1              | 11.5±1.5 | 0.121 | 0.850 |
| Large HDL particle (µM)          | 3.1±0.3           | 5.8±0.9               | 5.5±0.7 | 0.064 | 0.969 |
| Total Cholesterol Efflux Capacity (CEC %) | 11.6±0.4       | 13.8±1.2              | 13.2±1.2 | 0.241 | 0.623 |
| Non-ABCA1 specific CEC (%)      | 7.3±0.3           | 8.7±0.6               | 8.7±0.5 | 0.076 | 0.969 |
| ABCA1 specific CEC (%)          | 4.4±0.3           | 5.1±0.7               | 4.5±0.6 | 0.623 | 0.570 |

Values are expressed as mean ± SEM. p1: probability value obtained by Mann-Whitney U test of baseline values between NR and R. p2: probability value obtained by Wilcoxon test between standard statin therapy (baseline; SST) and half dose statin with supplements (HDS), for responders only.
Figure II 1: Baseline sterol profile (Mean ± SEM) of subgroups divided according to the intensity of LDL-C reduction (above or below the median; MED = -13%) after functional treatment. P values were obtained by T-test for independent groups. n=23 in each group.
Figure II 2: Spearman correlation graphs for Total Cholesterol Efflux Capacity vs HDL-C, Total HDL particle and CRP, obtained from baseline measures for responders (n=10) and non-responders (n=10).
CHEMICAL COMPOSITION AND OXIDATIVE STATUS OF FOOD COMPONENTS

Chocolate truffles ingredients were: cocoa powder, cocoa butter, cocoa liquor, palm oil, hazelnut paste, rice protein, polydextrose, erythriol, maltitol, flavor of hazelnuts, and soy lecithin.

The chemical composition of chocolates (determined according to the AOAC methods)[1] showed that daily consumption of functional and control truffles provided equal energy intake (91.80 ± 0.25 and 90.43 ± 0.55 Kcal, respectively). Chocolate truffles from functional treatment provided 1.10 ± 0.06 g of plant sterols/unit, while placebo truffles provided 0.11 ± 0.02 g of plant sterols/unit - quantified according to the method described by Laakso (2005) [2].

Softgels containing omega 3 fatty acids [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] were analyzed using gas chromatography (GC-MS)[3].

Fish oil provided 158.52 ± 3.89 mg of EPA/softgel and 89.78 ± 3.65 mg of DHA/softgel, which contributed to a daily intake of approximately 1.7 g of EPA+DHA. However, fish oil softgels were more oxidized than the placebo softgels, as determined by Thiobarbituric acid reactive substances (TBARS) and Peroxide Value methods [4,5]. Peroxide values were 10.74 ± 1.50 and 2.54 ± 0.11 meq/kg of oil for fish oil and placebo softgels, respectively (p<0.05). TBARS concentration of fish oil was 21.09 ± 3.48 mg TBARS/L of oil, while in placebo softgels concentration was 1.30 ± 0.10 mg TBARS/L of oil (p<0.05).

Tocopherols (Δ-tocopherol, γ-tocopherol and α-tocopherol) were also quantified in oil softgels, according to Gliszczynska-Świgło & Sikorska (2004)[6]. Placebo softgels provided higher total tocopherol intake (7.36 ± 0.02 mg/d, p <0.05) than fish oil treatment (6.84 ± 0.06 mg/d).

Epigallocatechin gallate (EGCG), the main polyphenol in green tea, was determined in tea samples according to Yoshida et al. (2007) [7]. EGCG concentration in green tea samples was 932.8 ± 143.7 µmol/L, which corresponded to an estimated intake of 170.8 mg EGCG/day. EGCG was not detected in anise tea samples.

Plasma fatty acids quantification

Plasma EPA, DHA and arachidonic acid (ARA, n-6) were measured using gas chromatography (GC-MS) as described by Shirai et al. (2005)[3]. Briefly, 150 µl of plasma were added to tubes containing internal standard (Tricosanoic Acid Methyl Ester (C23:0)). Afterward, 50 µL 0.5%
butylated hydroxytoluene (BHT) and 1 mL 0.5 M methanolic NaOH were added to all tubes. The solution was vortexed by 15 sec, and heated in water bath at 100°C/5 min. After cooling, samples were mixed with 2 mL methanol containing 14% boron trifluoride (BF₃) vortexed, and heated in water bath at 100°C for 5 min more. After cooling, 1 mL isooctane was added and the tubes were vigorously shaken for 30s. Saturated solution of NaCl (5 mL) was added and the tubes were gently homogenized. After centrifugation at 13,000 x g/5 min, the organic phase was transferred to a new vessel and dried under nitrogen stream. The recovered lipids were reconstituted in 0.5 mL isooctane. Fatty acids quantification was determined by gas chromatography (GC) equipped with a G3243A MS detector (Agilent 7890 A GC System, Agilent Technologies Inc., Santa Clara, USA). A fused silica capillary column (J&W DB-23 Agilent 122-236; 60 m x 250 mm inner diameter) was used for injection of 1 μL of sample. High-purity helium was used as the carrier gas at a flow rate of 1.3 ml/min with a split injection of 50:1. The oven temperature was programmed from 80°C to 175°C at a rate of 5°C/min, followed by another gradient of 3°C/min to 230°C, and kept at this temperature for 5 min. The temperature of GC inlet and transfer line were 250°C and 280°C, respectively. GC-MS was performed using 70 eV EI in scan acquisition and quantified by TIC. The fatty acids were identified by NIST11 comparing the retention times with those of four purified standard mixture of fatty acid methyl esters (Sigma Chemical Co.: 4-7801; 47085-U; 49453-U and 47885-U). All mass spectra were acquired over the m/z range of 40-500. Samples were analyzed in triplicate and results were expressed as mg or µg of each fatty acid/mL of plasma.

**Plasma sterols quantification**

Quantification of main plant sterols (campesterol and β-sitosterol) and cholesterol precursor (lathosterol) in plasma was performed according to García-Llatas et al. (2012)[8] using gas chromatography (GC-MS). Plasma aliquots (500 µl) were mixed with 2 µg of internal standard (5β-cholestan-3α-ol; Epicoprostanol) and evaporated to dryness through a nitrogen stream. A hot saponification was carried out by adding 500 µL of KOH 2.0 M in ethanol. After incubation (60°C/1h), each tube was added with MilliQ-water (400 µL) and hexane (2 µL), strongly shaken and centrifuged at 12,000 rpm for 5 min. The organic phase was transferred to another tube and all extraction process was repeated. Both supernatants (hexane phases) were combined and dried under nitrogen. The sterols were derivatized with 70 µL of bis(trimethylsilyl)-trifluoracetamid (BSTFA) containing 1% trimethylchlorosilane (TMCS) (99:1) and 50 µL of pyridine, at 70°C for 15 min. Trimethyl silyl ether (TMS) derivatives of plant sterols were separated on a fused silica capillary column coated with 5% phenyl/95% dimethylpolysiloxane (J&W HP-5 30 m x 0.32 mm i.d., film thickness 0.25 µm; Agilent Technologies Inc., Little Falls, DE, USA), with a GC equipped with a G3243A MS detector (Agilent 7890 A GC System, Agilent Technologies Inc., Santa Clara, USA).
An aliquot of 1.0 μL of derivatized sample solution was introduced into the column at 250°C, with a split ratio (1:50) injector and an autosampler. The components were separated at 300°C and detected with GC-MS at 280°C. The carrier gas was helium at flow rate of 1.0 ml/min. Reference standards: campesterol, β-sitosterol and lathosterol were purchased from Sigma (St. Louis, MO, USA) and used to identify the peaks. Quantification was calculated based on standard curve prepared with each sterol, using the area ratio sterol-IS, and results were expressed as μg/mg of cholesterol.

Plasma EGCG quantification was not possible due to extremely low concentrations that were beyond the sensitivity of detection.

**Plasma malondialdehyde (MDA)**

Malondialdehyde (MDA) concentration was assessed using reverse phase HPLC [9]. Plasma (0.05 mL) was submitted to alkaline hydrolysis with 12.5 μL of 0.2% BHT and 6.25 μL 10.0 M sodium hydroxide. This mixture was incubated at 60°C for 30 min and 750 μL of 0.44 mol/L trichloroacetic acid (TCA) containing 1% potassium iodide were added. The samples were kept on ice for 10 min and centrifuged (13,000 × g for 10 min). The supernatant (500 μL) was mixed with 250 μL of 0.6% thiobarbituric acid (TBA) and heated at 95°C for 30 min. After cooling, TBARS was extracted with 750 μL of n-butanol, and 50 μL was analyzed by HPLC (Agilent Technologies 1200 Series; Santa Clara, CA, USA). The TBA-MDA conjugate derivative was injected into a Phenomenex reverse-phase C18 analytical column (250 mm × 4.6 mm; 5 μm, Phenomenex, Torrance, CA, USA) with a LC8-D8 pre-column (Phenomenex AJ0-1287) and was fluorometrically quantified at excitation of 515 nm and emission of 535 nm. The HPLC pump delivered the isocratic mobile phase: 60% phosphate buffered saline (PBS) (50 mmol/L, pH 7.1) + 40% methanol at a flow rate of 1.0 mL/min. A standard curve (0.5 to 20 μmol/L, r = 0.995) was prepared using 1,1,3,3-tetraethoxypropane (Sigma, T9889, Cambridge, MA, USA).

**Antioxidant enzyme activity of erythrocyte lysates**

The superoxide dismutase (SOD) activity of erythrocyte lysates was determined using a microassay based on an aerobic reaction between NADH and phenazinemethosulphate (PMS), that generates superoxide anion radical (O2−) under nonacidic pH [10]. The nitro blue tetrazolium (NBT) was used as detector molecule to assess O2− dismutation by tissue SODs (Cu-, Zn- and Mn-type), through reduction to a stable formazan product. Samples (25 μL) were placed into a 96 well microplate and 200 μL of freshly prepared solution containing 0.1 mmol/L ethylenediamine tetraacetic acid (EDTA), 62 μmol/L NBT and 98 μmol/L NADH in 50 mmol/L phosphate buffer (pH 7.4) were
added. The reaction was initiated with the addition of 25 μL freshly prepared 33 μmol/L PMS in 50 mmol/L phosphate buffer (pH 7.4) with 0.1 mmol/L EDTA. The reaction was monitored for 5 min at 560 nm at 26°C, by a multi-detection microplate reader (BioTek, Synergy HT, Winooski, VT, USA). The SOD scavenging of O$_2^-$ effect (U/mL) was calculated by interpolation of the inhibition (%) of the formazan formation using a linear regression (r = 0.994) prepared with 2–7 U/mL of SOD from bovine erythrocytes (Sigma, S7446, Cambridge, MA, USA). For the catalase (CAT) activity determination [11], erythrocyte lysate was placed into a 96-well microplate with UV treatment, added to 140 μL of phosphate buffer (50 mmol/L with 0.1 mmol/L EDTA, pH 7.4). The reaction was started by adding 40 μL of 30 mmol/L hydrogen peroxide, freshly prepared in the same phosphate buffer. The absorbance at 240 nm was continuously monitored over 8 min at 30°C. The CAT activity was expressed in U/mL, calculated by a linear regression (r = 0.9944) using the % of inhibition of hydrogen peroxide decomposition promoted by catalase at 2–10 U/mL (Sigma Chemical Co., St. Louis, MO, USA). To determine glutathione peroxidase (GPx) activity [12], erythrocyte lysate (containing 4 mg/mL of protein) was incubated with 125 μmol/L 0.1 M phosphate buffer containing 1 mmol/L EDTA (pH 7.4), 5 μL freshly prepared 0.08 mol/L reduced glutathione and 5 μL freshly prepared glutathione reductase (9.6 U) at 37 °C for 5 min, in a 96-well microplate. Then 30 μL of 1.2 mmol/L NADPH and 5 μL of 0.46% tert-butylhydroperoxide were added to the solution. The absorbance at 340 nm was continuously monitored over 4 min at 37°C. The GPx activity was expressed in U/mL, calculated by a linear regression (r = 0.998) using the % of inhibition promoted by glutathione peroxidase at 0.15–1.35 U/mL of (Sigma Chemical Co.). Determination of glutathione reductase (GR) activity [13] was performed by incubation of the erythrocyte lysate containing 10 mg/mL of protein with 190 μL reaction medium (2 mL of 0.1 mol/L phosphate buffer pH 7 with 1 mmol/L EDTA, 1.5 mL of 0.005 mol/L EDTA, 1.5 mL of milli-Q water, 10 mg of glutathione disulphide and 2 mg NADPH). The absorbance at 340 nm was continuously monitored over 6 min at 37°C. The GR activity (U/mL) was calculated by a linear regression (r = 0.9909) using the % of inhibition of NADPH oxidation promoted by glutathione reductase at 0.0003–0.25 U/mL (Sigma Chemical Co.).

**Cholesterol Efflux Capacity**

Cholesterol efflux capacity was quantified according to the method created by Rader and Rothblat [14]. J774 cells (murine macrophage cell line) were plated and radiolabeled with 2 μCi of $^3$H-cholesterol/mL. ABCA1 was up-regulated by incubation with 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP for 6-hours. Subsequently, efflux mediums containing 2.8% apolipoprotein B–depleted serum were added for 4 hours. All steps were performed in the presence of the acyl–coenzyme A:cholesterol acyltransferase inhibitor CP113,818 (2 μg/mL). Liquid scintillation counting was
used to quantify the efflux of radioactive cholesterol from the cells. The quantity of radioactive cholesterol incorporated into cellular lipids was calculated by means of isopropanol extraction of control wells not exposed to patient serum. Percent efflux was calculated by the following formula:

\[
\frac{\text{microcuries of } ^3\text{H}-\text{cholesterol in mediums containing 2.8\% apolipoprotein B–depleted serum–microcuries of } ^3\text{H}-\text{cholesterol in serum-free mediums)}}{\text{microcuries of } ^3\text{H}-\text{cholesterol in cells extracted before the efflux step}}} \times 100.
\]

All assays were performed in duplicate. To correct for interassay variation across plates, a pooled serum control from five healthy volunteers was included on each plate, and values for serum samples from patients were normalized to this pooled value in subsequent analyses.

References

[1] AOAC. Official methods of analysis of AOAC. AOAC Int (18th Ed) Gaithersburg, MD 2005.

[2] Laakso P. Analysis of sterols from various food matrices. Eur J Lipid Sci Technol 2005;107:402–10. doi:10.1002/ ejlt.200501134.

[3] Shirai N, Suzuki H, Wada S. Direct methylation from mouse plasma and from liver and brain homogenates. Anal Biochem 2005;343:48–53. doi:10.1016/j.ab.2005.04.037.

[4] McDonald RE, Hultin H. Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. J Food Sci 1987;52:15–21.

[5] Shantha NC, Decker EA. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. J AOAC Int 1994;77:421–4.

[6] Gliszczyńska-Świgło A, Sikorska E. Simple reversed-phase liquid chromatography method for determination of tocopherols in edible plant oils. J Chromatogr A 2004;1048:195–8. doi:10.1016/j.chroma.2004.07.051.

[7] Yoshida T, Majors RE, Kumagai H. High-Speed Analyses using Rapid Resolution Liquid Chromatography on ZORBAX column packed 1.8 µm Particles. Chromatography 2007;28:81–7.

[8] García-Llatas G, Vidal C, Cilla A, Barberá R, Lagarda MJ. Simultaneous quantification of serum phytosterols and cholesterol precursors using a simple gas chromatographic method. Eur J Lipid Sci Technol 2012;114:520–6. doi:10.1002/ ejlt.201100331.

[9] Hong Y, Yeh S, Chang C, Hu M. Total Plasma Malondialdehyde Levels in 16 Taiwanese College Students Determined by Various Thiobarbituric Acid Tests and an Improved High-Performance Liquid Chromatography-based Method. Clin Biochem 2000;33:619–25.
[10] Ewing JF, Janero DR. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anal Biochem 1995;232:243–8. doi:10.1006/abio.1995.0014.

[11] Nabavi SF, Habtemariam S, Sureda A, Hajizadeh Moghaddam A, Daglia M, Nabavi SM. In vivo protective effects of gallic acid isolated from Peltiphyllum peltatum against sodium fluoride-induced oxidative stress in rat erythrocytes. Mol Cell Biochem 2013;372:233–9.

[12] Flohé L, Günzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984;105:114–20.

[13] Torres LL, Quaglio NB, de Souza GT, Garcia RT, Dati LMM, Moreira WL, et al. Peripheral oxidative stress biomarkers in mild cognitive impairment and Alzheimer’s disease. J Alzheimer’s Dis 2011;26:59–68. doi:10.3233/JAD-2011-110284.

[14] De La Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. Arterioscler Thromb Vasc Biol 2010;30:796–801. doi:10.1161/ATVBAHA.109.199158.
SUPPLEMENTARY FIGURES

**Figure II S1:** Study control markers, before and after each treatment. (A) Mean EPA+DHA/ARA ratio (B) Mean β-sitosterol:cholesterol ratio. *P* values were obtained by T-test. FNT: functional treatment; CTR: control treatment.

**Figure II S2:** Mean changes in plasma EPA+DHA concentration by quartiles according to baseline EPA+DHA values. Q1: 0-33.9 μg/mL (n=13); Q2: 37.5-54.2 μg/mL (n=13); Q3: 54.2-72.7 μg/mL (n=13); Q4: 73.17-120.6 μg/mL (n=13). *P* values were obtained by T-test for independent groups. FNT: functional treatment; CTR: control treatment.
Figure II S3: Mean % change of TG and MDA in subgroups of patients according to baseline median. A1. Baseline TG above the median (93 mg/dL) (n=23). A2. Baseline TG below the median (n=21). B1. Baseline MDA above the median (2.23 µmol/L) (n=23). B2. Baseline MDA below the median (n=18). *P* values were obtained by T-test for independent groups. FNT: functional treatment; CTR: control treatment.

Figure II S4: Mean % change of LDL and CRP for Responders (R; n=10) and Non-Responders (NR; n=10) after 6 weeks of functional treatment, at the end of the first phase of the study. *P* values were obtained by Mann-Whitney test.
*Figure II S5:* Spearman correlation graphs obtained from baseline measures for responders (n=10) and non-responders (n=10).
HDL-C has long been known as the “good cholesterol”, once it helps remove excess cholesterol from the arteries and circulation. As a high level of HDL-C is associated with increased protection against CVD, it should be simple: as higher as HDL-C gets, more protected from heart attacks we are. And then the HDL hypothesis started taking some hits: raising HDL levels with drugs had disappointing results and mutations that are responsible for increasing HDL-C were shown to be actually associated with increased risk for heart disease. The complexity of HDL composition and biology led researchers in the field to redefine HDL hypothesis towards the concept of HDL functionality: beneficial properties of HDL particles, independent of cholesterol levels.

The following chapter describes activities performed during a research internship carried out at the New York University School of Medicine, Division of Cardiology, under the supervision of Dr. Edward A. Fisher, Director of Marc and Ruti Bell Program in Vascular Biology and The Center for the Prevention of Cardiovascular Disease.

Despite our efforts, science is never straightforward. Challenges and methodological issues encountered during the course of experiments, while pursuing assays of HDL functionality, are discussed herein.
Effects of a combined bioactive supplement on HDL function in statin-treated patients

Introduction

While LDL-C is an established risk factor for cardiovascular disease (CVD), epidemiological studies have inversely associated HDL-C levels to cardiovascular outcomes, as an independent risk factor (Castelli et al., 1986; Franceschini, 2001). The cardioprotective effects of HDL particle have been associated with its function in reverse cholesterol transport (RCT) and to anti-inflammatory, antithrombotic, antioxidant effects and improvement of endothelial function (Feig, Hewing, Smith, Hazen, & Fisher, 2014). Whereas it would seem that by increasing HDL-C, the cardiovascular risk would be decreased, clinical trials with HDL-C raising therapies have failed to demonstrate such association (Kaur et al., 2014). Although the most recent study on CETP-inhibitors showed a reduction on major coronary events in high-risk patients, the increase in HDL-C did not appear to have much impact on risk reduction (The HPS3/TIMI55-REVEAL Collaborative Group, 2017).

These contradictory findings brought attention to the importance of the distinction between HDL particles functionality and plasma HDL-C (Feig et al., 2014) and highlighted the concept that measures of HDL parameters in serum do not always reflect HDL function (De La Llera-Moya et al., 2010). The main atheroprotective function attributed to HDL is the ability to promote macrophage cholesterol efflux, a critical initial step in RCT, which contributes to unload excess of cholesterol from artery wall (Fisher, Feig, Hewing, Hazen, & Smith, 2012).

However, published data show that neither measures of HDL-C nor measures of serum apoA-I are good predictors of the capacity of individual sera to promote efflux from macrophages (De La Llera-Moya et al., 2010). This is partially explained by the fact that apoA-I is a selective target for oxidative modification by myeloperoxidase, which can significantly impair ATP-binding cassette A1 (ABCA1)-mediated efflux (Zheng et al., 2004). In fact, apoA1 recovered from human atheroma was shown to be highly oxidized and presenting dysfunctional cholesterol efflux activity (DiDonato et al., 2013; Huang et al., 2014).

There is increasing consensus that measures of HDL functionality may be useful as diagnostic and criterion to identify patients under cardiovascular risk, as well as to select the specific drug therapy (Fisher et al., 2012). Moreover, HDL functionality measure could be a better predictor of cardiovascular risk than HDL-cholesterol levels. A retrospective study reported that HDL ability to
promote cholesterol efflux from macrophages was strongly and inversely associated with both subclinical atherosclerosis and obstructive coronary artery disease (Rodrigues et al., 2011). Likewise, a prospective study involving healthy volunteers observed that incident atherosclerotic cardiovascular disease was also inversely associated with cholesterol efflux (Rohatgi et al., 2014).

Therefore, it has been proposed that more important than simply increasing HDL-C it would be to increase HDL functionality (Feig et al., 2014). Considering that HDL loss of function is associated to ApoA-1 oxidation (Fisher et al., 2012; Hewing et al., 2014), composition of HDL lipid core and surface lipids properties (Kontush, Lhomme, & Chapman, 2013), we hypothesized that a combined bioactive supplementation that provides antioxidant, anti-inflammatory and lipid lowering activity could ameliorate or increase HDL functionality in statin-treated patients.

To evaluate HDL functionality in patient’s samples, our primary aim was to develop new methodologies that accurately measure HDL function and that ultimately could be brought to high-throughput for clinical purposes. Well established techniques, such as Cholesterol Efflux Capacity (CEC) and HDL particle size measurement were readily applied to the samples.

**Optimization of HDL isolation protocol**

When isolating HDL by ultracentrifugation from reduced sample volumes (i.e 1 mL of plasma), albumin contamination of the HDL fraction may occur (Figure 1). Therefore, the first step in pursuing assays of HDL functionality was to optimize the density gradient ultracentrifugation method (Chapman, Goldstein, Lagrange, & Laplaud, 1981). Final protocol is attached (page 86).

![Figure III 1: HDL isolation technique. (A) Total protein staining (Ponceau; n=3 independent samples) and (B) Western Blot for human ApoA1 and human Albumin in HDL fractions isolated from 1 mL of human plasma.](image-url)
Optimized protocol for HDL isolation is shown in panel C (n=4 independent samples) without albumin contamination, as shown by total protein staining.

**HDL anti-inflammatory activity: Macrophage polarization experiments**

Macrophages have long been known to play crucial roles in atherosclerotic plaque progression and regression. Previous studies from Dr. Fisher’s lab have shown that HDL can lead to a shift in macrophage phenotype from an inflammatory state (M1) towards an anti-inflammatory state (M2) in vivo and in vitro (Feig et al., 2011; Sanson, Distel, & Fisher, 2013), and that polarization to the M2 state is required for resolution of atherosclerotic inflammation and plaque regression (Rahman et al., 2017). The published method for the in vitro macrophage polarization assay was replicated with adaptations, as described below.

Bone marrow-derived macrophages (BMDM) were isolated from the femur and tibia of adult mice. Cells were pooled in PBS, and centrifuged at 1200 rpm, for 5 min, at room temperature. After blood cell lysis (RLB, Sigma-Aldrich) cells were plated in 6 well plates (12 wells per mice) in DMEM (1g/L glucose) containing 10% FBS and 10ng/mL M-CSF. Fresh media was added on day 4. Cells were treated on days 5-6. For M2 polarization experiments, cells were initially treated with commercial HDL (Kalen Biomedical, LLC) from 50 to 400 ug/mL for 24h, with and without IL-4 (0.1 and 1 ng/mL) for the following 24h. For M1 downregulation studies, IFNγ (20 ng/mL) was added to the wells after 24h of HDL treatment.

Total RNA was isolated from the BMDMs using TRIzol (Invitrogen) and purified using Directzol RNA Miniprep kit (Zymo Research). Total RNA was reverse transcripted using the Verso cDNA kit (Thermo Scientific). Real-time PCR was performed using the ABI PRISM 7300 sequence detection system (Applied Biosystems). Gene expression was normalized to HPRT expression and assessed using the Δ(ΔCt) calculation method.

Results obtained from M2 polarization experiments are shown in Figure 2 and 3. HDL treatment alone was not effective in increasing expression of M2 markers (Arginase 1 and Mannose receptor). Co-treatment with IL-4 0.1 ng/mL and HDL at higher doses (200 and 400 ug/mL) showed a
better response in terms of Arginase 1 enhancement, with high deviation (Figure 2). However, when the experiment was repeated with fresh isolated BMDMs (Figure 3), co-treatment with IL-4 1 ng/mL and HDL 400 ug/mL was more effective in increasing Arginase 1 and Mannose Receptor expression, with no observed effect at 0.1 ng/mL of IL-4. M2 polarization experiments were also tested with RAW cells and immortalized BMDMs, with no effect being observed (data not shown).

Results obtained from M1 downregulation experiments are shown in Figure 4. Inhibition of inflammatory markers was more pronounced at the higher tested dose of HDL (400 ug/mL) (Figure 4A). However, cells at resting state (M0) have an extremely low expression of inflammatory genes. Therefore, cells were challenged with IFNy after 24h of HDL pre-treatment (Figure 4B). HDL pre-treatment seemed to downregulate inflammation, although not for all three M1 markers.

Overall, higher HDL doses (200 and 400 ug/mL) were more effective, unlike previously shown - 50 ug/mL, in the original study (Sanson et al., 2013). These concentrations would translate into a large amount of isolated HDL from human plasma to be used for one single assay. Due to the limited availability of samples and low consistency of observed effects, individual human samples from our clinical study were not tested for macrophage polarization.
Figure III 2: Gene expression of M2 markers (Arginase 1 and Mannose receptor) in primary macrophages pre-treated with HDL for 24h, followed by IL-4 treatment (24h). Results from 2 independent experiments with fresh isolated BMDMs, in duplicate. *P<0.05, ANOVA.
Figure III 3: M2 polarization experiment was repeated in primary macrophages only for higher HDL doses (200 and 400 ug/mL). One experiment, in duplicate.
Figure III 4: M1 downregulation experiments. (A) Expression of proinflammatory genes (MCP1, TNFα and iNOS) was quantified in BMDMs after treating cells for 48h with HDL at doses of 50, 100, 200 and 400 ug/mL. Mean of 2 independent experiments, in duplicate. (B) Cells were pre-treated with HDL for 24h prior to IFNγ stimulus (20 ng/mL). Mean of 3 independent experiments, in duplicate. * P<0.05, ANOVA.
Foam-like Smooth Muscle Cells (SMC) phenotypic reversal

Over a decade ago, the Fisher Lab was the first to report that vascular SMC transdifferentiate to macrophage-like foam cells when loaded with cholesterol (Rong, Shapiro, Trogan, & Fisher, 2003). Other research groups have recently observed that more than 50% of foam cells in advanced human coronary artery plaques are of SMC origin and express substantially less ABCA1 compared to foam cells from a macrophage origin (Allahverdian, Chehroudi, Mcmanus, Abraham, & Francis, 2014). Studies from the Fisher Lab have shown that HDL is able to mediate cholesterol efflux from SMCs and to induce phenotypic reversal of cholesterol-laden SMCs – reducing macrophage characteristics and restoring an SMC phenotype (Vengrenyuk et al., 2015). Whether the phenotypic reversal is dependent upon the efficacy of cholesterol efflux from these cells by HDL, or another property of the lipoprotein particle, is unknown. Therefore, measuring the ability of HDL to reverse and restore an SMC phenotype could be a new assay of HDL functionality, as it presumably contributes to atherosclerosis regression and plaque stabilization.

Human Coronary Artery Smooth Muscle Cells (HCASMC) were purchased from Cell Applications Inc. Cells with passage number < 10 were used in the described experiments. Cells were grown in complete Human Smooth Muscle Cell Growth Medium (Cell Applications Inc.). HCASMC were loaded with cholesterol by using Cholesterol–methyl-β-cyclodextrin complex (Cholesterol-Water Soluble, #C4951, Sigma). After 24h of serum starvation (DMEM 0.2% BSA), subconfluent cells were incubated with 10 ug/mL of cholesterol in DMEM 0.2% BSA, for 72h. Fresh starvation media was added to control wells. Treatment cytotoxicity was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) after treating cells for 72h with 5, 10 and 20 ug/mL cholesterol (Figure 5). RNA isolation and Real-time PCR was performed as described before.

Figure III 5: Cytotoxicity of cholesterol treatment after 72h.
Cholesterol loading of HCASMCs was previously shown to reduce the SMC phenotype and contractile function (reduced expression of α-actin) and increase the expression of macrophage genes (as ABCA1 and CD68) (Rong, Shapiro, Trogan, & Fisher, 2003). Figure 6 shows the results of 4 attempts to reproduce the macrophage-like phenotype, without success. To unveil what the issue was, the experiment was repeated twice again, comparing cells from different companies (Figure 7). As results were again not convincing, minor changes were made to the protocol (increased starvation time and different media). At last, Figure 8 shows the expected phenotype after loading SMC with cholesterol. As the internship came to an end by the time we got a working protocol, the effect of commercial HDL in reversing this phenotype, compared to the ability of patient’s samples (before and after treatments) to do the same, could not be assessed.

**Figure III 6**: Gene expression of SMC marker (α-Actin) and macrophage markers (ABCA1 and CD68) after loading HCASMC with cholesterol (10 ug/mL). Results are representative of 4 independent experiments, in triplicate. *P<0.05, T-test.

**Figure III 7**: Gene expression of SMC marker (α-Actin) and macrophage markers (ABCA1 and CD68) after loading HCASMC with cholesterol (10 ug/mL). Comparison between cells purchased from different companies
(Lonza and Cell Applications). Results are representative of 2 independent experiments, in triplicate. *P<0.05, ANOVA.

![Figure 3](image)

**Figure III 8:** Gene expression of SMC marker (α-Actin) and macrophage markers (ABCA1 and CD68) after loading HCASMC with cholesterol (10 μg/mL), after minor changes in the protocol. Results are representative of 2 independent experiments, in triplicate.

**Cholesterol Efflux capacity (CEC) and HDL particle size in human samples**

The results of the first phase of our clinical study demonstrated that plasma LDL-C and hs-CRP were significantly reduced by the supplementation (n=53). However, triglycerides and MDA were reduced only in a subgroup of patients with baseline values above the median (n=23). In order to proceed to second phase (pilot study of statin dose reduction), we chose to select patients that exhibited a response to supplementation in each component of inflammation, lipemia, and oxidative stress. Results from the second phase showed no significant change in LDL-C, HDL-C, VLDL-C, triglycerides, and hs-CRP (n =10) after statin dose reduction by 50%, in a supplementation background, compared to baseline values (100% statin) - demonstrating the potential of statin dose reduction by complementary diet therapy. To further investigate the effects of this new therapy, we have turned our attention to HDL functionality. As patients initially completed 6 weeks of functional treatment before having statin dose reduced, we could also investigate the effects of supplementation on HDL functionality.

CEC and HDL particle size were determined as mentioned in Chapter 2, with the collaboration of Dr. Tomás Vaiser (University of Washington, Seattle, US). **Figure 9** shows the average profile of HDL size peaks. Currently, there is no consensus on the clinical relevance of circulating concentrations
of different HDL subparticles (Fazio & Pamir, 2016; Kontush, 2015). Large HDL particles have been negatively associated with cardiovascular risk (Kontush, 2015) and a mean HDL size higher than 8.2 nm was shown to be more effective than high HDL-C plasma concentration in predicting subclinical carotid atherosclerosis in low risk individuals (Parra et al., 2014). Recently, patients with coronary endothelial dysfunction were shown to have decreased number of large HDL particles, compared to control subjects, which may contribute to impaired CEC observed for those patients (Monette et al., 2016). Another study showed that lipid-poor and small HDL displayed both high capacity for ABCA1-mediated cholesterol efflux and strong anti-inflammatory action, whereas the larger particles showed stronger antioxidant function (Didichenko et al., 2016).

These findings suggest that “the right mix” of multiple HDL particle sizes may be more effective for cardioprotective benefits, although further research is necessary to elucidate the function and compositional signature of HDL subparticles and how it may be affected by pharmacological or dietary interventions (Fazio & Pamir, 2016).

**Figure III 9:** Average scan for human HDL particle size distribution. In general, 4 subspecies are detected in human HDL: extra-small, small, medium and large. HDL particles were quantified by calibrated ion mobility analysis (IMA), which separates nanoparticles by size in the gas phase.

**Figures 10 and 11** show that CEC and HDL particle size distribution of patient’s plasma did not change after treatments. Over a decade ago, a study showed that CEC was not affected by different
types of dietary fatty acids (8.3% trans vs. 14.6% polyunsaturated vs. 13.2% saturated) (Buonacorso et al., 2007). Recently, Mediterranean diet enriched with olive oil was shown to increase CEC compared to baseline values, possibly through the improvement of HDL particles antioxidant status and lipid composition (Hernáez et al., 2017). These authors observed a decrease in the triglyceride content in HDL core and an increase in surface phospholipids, which is linked to greater fluidity (Hernáez et al., 2017). The same research group had previously shown that consumption of polyphenol-rich olive oil promotes a triglyceride-poor core, increases HDL size and CEC, and enhances HDL oxidative status, which was associated with an increase in the lipoprotein content of olive oil polyphenols (Hernáez et al., 2014). CEC and antioxidant activity of HDL were also shown to increase after dietary supplementation with 1800 mg/day of EPA, in patients with coronary risk factors (Tanaka, Ishida, Nagao, & Mori, 2014).

As this was a pilot study (n=10), further investigation (and the inclusion of placebo control group) would be needed to conclude if our proposed supplementation can affect CEC or other HDL function.

**Figure III 10:** Cholesterol efflux capacity of human samples according to treatments: baseline (100% statin), after 6 weeks of supplementation and after 50% statin dose reduction in a supplementation background. *P<0.05, ANOVA.
Figure III 11: HDL particle size distribution in human plasma according to treatments: baseline (100% statin), after 6 weeks of supplementation and after 50% statin dose reduction in a supplementation background. *P<0.05, ANOVA.
References

Allahverdian, S., Chehroudi, A.C., Mcmanus, B.M., Abraham, T., and Francis, G.A. (2014). Contribution of Intimal Smooth Muscle Cells to Cholesterol Accumulation and Macrophage-Like Cells in Human Atherosclerosis. *Circulation*, 129, 1551–1559.

Buonacorso, V., Nakandakare, E.R., Nunes, V.S., Passarelli, M., Quinta, E.C.R., and Lottenberg, A.M.P. (2007). Macrophage cholesterol efflux elicited by human total plasma and by HDL subfractions is not affected by different types of dietary. *American Journal of Clinical Nutrition*, 86, 1270–1277.

Castelli, W.P., Garrison, R.J., Wilson, P.W., Abbott, R.D., Kalsoudian, S., and Kannel, W.B. (1986). Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. *JAMA: the journal of the American Medical Association*, 256 (20), 2835–2838.

Chapman, M.J., Goldstein, S., Lagrange, D., and Laplaud, P.M. (1981). A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *Journal of Lipid Research*, 22, 339–358.

Didichenko, S.A., Navdaev, A. V, Cukier, A.M.O., Gille, A., Schuetz, P., Spycher, M.O., Thérond, P., Chapman, M.J., Kontush, A., and Wright, S.D. (2016). Integrative Physiology Enhanced HDL Functionality in Small HDL Species Produced Upon Remodeling of HDL by Reconstituted. *Circulation Research*, 119, 751–763.

DiDonato, J. a., Huang, Y., Aulak, K.S., Even-Or, O., Gerstenecker, G., Gogonea, V., Wu, Y., Fox, P.L., Tang, W.H.W., Plow, E.F., Smith, J.D., Fisher, E. A., and Hazen, S.L. (2013). Function and distribution of apolipoprotein A1 in the artery wall are markedly distinct from those in plasma. *Circulation*, 128, 1644–1655.

Fazio, S. and Pamir, N. (2016). HDL Particle Size and Functional Heterogeneity. *Circulation Research*, 119, 704–708.

Feig, J.E., Hewing, B., Smith, J.D., Hazen, S.L., and Fisher, E.A. (2014). High-density lipoprotein and atherosclerosis regression: Evidence from preclinical and clinical studies. *Circulation Research*, 114, 205–213.

Feig, J.E., Rong, J.X., Sanson, M., Vengrenyuk, Y., Liu, J., Moore, K., Garabedian, M., Edward, A., Feig, J.E., Rong, J.X., Shamir, R., Sanson, M., Vengrenyuk, Y., and Liu, J. (2011). HDL promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells. *PNAS*, 108, 7166–7171.

Fisher, E.A., Feig, J.E., Hewing, B., Hazen, S.L., and Smith, J.D. (2012). High-density lipoprotein function, dysfunction, and reverse cholesterol transport. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 32, 2813–2820.
Franceschini, G. (2001). Epidemiologic evidence for high-density lipoprotein cholesterol as a risk factor for coronary artery disease. *The American journal of cardiology*, 88, 9N–13N.

Hernández, Á., Castañer, O., Elosua, R., Pintó, X., Estruch, R., Salas-Salavador, J., Corella, D., Arós, F., Serra-Majem, L., Fiol, M., Ortega-Calvo, M., Ros, E., Martínez-González, M.Á., de la Torre, R., López-Sabater, M.C., and Fitó, M. (2017). Mediterranean Diet Improves High-Density Lipoprotein Function in High-Cardiovascular-Risk Individuals. *Circulation*, 135, 633–643.

Hernández, Á., Fernández-castillejo, S., Farràs, M., Catalán, Ú., Subirana, I., Montes, R., Solà, R., Muñoz-aguayo, D., Gelabert-gorgues, A., Díaz-gil, Ó., Nyyssönen, K., Zunft, H.F., Torre, R. De, Martín-peláez, S., Pedret, A., Remaley, A.T., and Covas, M. (2014). Olive Oil Polyphenols Enhance High-Density Lipoprotein Function in Humans. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34, 2115–2119.

Hewing, B., Parathath, S., Barrett, T., Ki, W., Chung, K., Astudillo, Y.M., Hamada, T., Ramkhelawon, B., Tallant, T.C., Yusufishaq, M.S.S., Didonato, J.A., Huang, Y., Buffa, J., Berisha, S.Z., Smith, J.D., Hazen, S.L., and Fisher, E.A. (2014). Effects of Native and Myeloperoxidase-Modified Apolipoprotein A-I on Reverse Cholesterol Transport and Atherosclerosis in Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34, 779–789.

Huang, Y., Didonato, J.A., Levison, B.S., Schmitt, D., Li, L., Wu, Y., Buffa, J., Kim, T., Gerstenecker, G.S., Gu, X., Kadiyala, C.S., Wang, Z., Culley, M.K., Hazen, J.E., Didonato, A.J., Fu, X., Berisha, S.Z., Peng, D., Nguyen, T.T., Liang, S., Chuang, C., Cho, L., Plow, E.F., Fox, P.L., Gogonea, V., Tang, W.H.W., Parks, J.S., Fisher, E.A., Smith, J.D., and Hazen, S.L. (2014). An abundant dysfunctional apolipoprotein A1 in human atheroma. *Nature Medicine*, 20, 193–206.

Kaur, N., Pandey, A., Negi, H., Shafiq, N., Reddy, S., Kaur, H., Chadha, N., and Malhotra, S., (2014). Effect of HDL-raising drugs on cardiovascular outcomes: A systematic review and meta-regression. *PLoS ONE*, 9 (4), 1–11.

Kontush, A. (2015). HDL particle number and size as predictors of cardiovascular disease. *Frontiers in Pharmacology*, 6, 1–6.

Kontush, A., Lhomme, M., and Chapman, M.J. (2013). Unraveling the complexities of the HDL lipidome. *Journal of lipid research*, 54, 2950–63.

De La Llera-Moya, M., Drazul-Schrader, D., Asztalos, B.F., Cuchel, M., Rader, D.J., and Rothblat, G.H. (2010). The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 30, 796–801.

Monette, J.S., Hutchins, P.M., Ronsein, G.E., Wimberger, J., Irwin, A.D., Tang, C., Sara, J.D.,
Shao, B., Vaisar, T., Lerman, A., and Heinecke, J.W. (2016). Patients With Coronary Endothelial Dysfunction Have Impaired Cholesterol Efflux Capacity and Reduced HDL Particle Concentration. *Circulation Research*, 119, 83–91.

Parra, E.S., Panzoldo, N.B., De Zago, V.H.S., Scherrer, D.Z., Alexandre, F., Bakkarat, J., Nunes, V.S., Nakandakare, E.R., Quintão, E.C.R., Nadruz-, W., De Faria, E.C., and Sposito, A.C. (2014). HDL size is more accurate than HDL cholesterol to predict carotid subclinical atherosclerosis in individuals classified as low cardiovascular risk. *PLoS ONE*, 9 (12), 1–12.

Rahman, K., Vengrenyuk, Y., Ramsey, S.A., Vila, N.R., Girgis, N.M., Liu, J., Gusarova, V., Gromada, J., Weinstock, A., Moore, K.J., Loke, P., and Fisher, E.A. (2017). Inflammatory Ly6C hi monocytes and their conversion to M2 macrophages drive atherosclerosis regression. *The Journal of Clinical Investigation*, 127, 2904–2915.

Rodrigues, A., Burke, M.F., Jafri, K., French, B.C., Ph, D., Phillips, J.A., Ph, D., Mucksavage, M.L., Sc, M., Wilensky, R.L., Mohler, E.R., Rothblat, G.H., Ph, D., and Rader, D.J. (2011). Cholesterol Efflux Capacity, High-Density Lipoprotein Function, and Atherosclerosis. *The New England Journal of Medicine*, 364, 127–135.

Rohatgi, A., Khera, A., Berry, J.D., Givens, E.G., Ayers, C.R., Wedin, K.E., Neeland, I.J., Yuhanna, I.S., Rader, D.R., Lemos, J. A. De, and Shaul, P.W. (2014). HDL cholesterol efflux capacity and incident cardiovascular events. *The New England Journal of Medicine*, 317, 2383–93.

Rong, J.X., Shapiro, M., Trogan, E., and Fisher, E.A. (2003). Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. *PNAS*, 100, 13531–13536.

Sanson, M., Distel, E., and Fisher, E.A. (2013). HDL Induces the Expression of the M2 Macrophage Markers Arginase 1 and Fizz-1 in a STAT6-Dependent Process. *PLoS ONE*, (8), e74676.

Tanaka, N., Ishida, T., Nagao, M., and Mori, T. (2014). Administration of high dose eicosapentaenoic acid enhances anti-inflammatory properties of high-density lipoprotein in Japanese patients with dyslipidemia. *Atherosclerosis*, 237, 577–583.

The HPS3/TIMI55–REVEAL Group (2017). Effects of Anacetrapib in Patients with Atherosclerotic Vascular Disease. *New England Journal of Medicine*, published online in August (29).

Vengrenyuk, Y., Nishi, H., Long, X., Ouimet, M., Savji, N., Martinez, F.O., Cassella, C.P., Moore, K.J., Ramsey, S.A., Miano, J.M., and Fisher, E.A. (2015). Myocardin Axis to Convert Aortic Smooth Muscle Cells to a Dysfunctional Macrophage-Like Phenotype. *Atherosclerosis, Thrombosis, and Vascular Biology*, 35, 535–546.
Zheng, L., Nukuna, B., Brennan, M.L., Sun, M., Goormastic, M., Settle, M., Schmitt, D., Fu, X., Thomson, L., Fox, P.L., Ischiropoulos, H., Smith, J.D., Kinter, M., and Hazen, S.L. (2004). Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and function impairment in subjects with cardiovascular disease. *Journal of Clinical Investigation*, 114 (2), 529–541.
Drug treatment for dyslipidemia is unquestionably effective in reducing overall cardiovascular events. However, current therapies have many drawbacks and still do not achieve complete cardiovascular protection. This residual risk brought attention to the need of new therapies and clinical targets beyond LDL-C, especially as the atherosclerotic process also involves secondary lipid parameters, inflammation, and oxidative stress. The potential of pharmacological and/or dietary interventions to modulate HDL functionality is also an attractive field and a hot topic, though further studies are necessary to unravel the complexity of HDL biology.

In this sense, the combination of bioactive compounds with drugs appears to be a promising therapy to reduce CVD progression. Our findings show the potential cardiovascular improvement by including omega 3 fatty acids, plant sterols and polyphenols as drugs co-therapy, for primary prevention. The co-therapy proposed in our study represents a simple, effective and low-cost strategy that could improve life quality of patients that are poor responders or those who suffer from adverse side effects associated with high doses of statins. However, long-term clinical trials are necessary to establish if this alternative therapy could promote a reduction of cardiovascular events.

We have also observed different patterns of response towards supplementation, which highlights the potential of personalized medicine in enhance patient’s care and therapy success. This personalized approach could especially benefit statin-intolerant patients who have suboptimal LDL-C management yet cannot tolerate increases in satin dosage. The alternative treatment option of combining nutraceuticals to reduced satin dosage could significantly increase adherence to long-term treatment and is less expensive than other add-on therapies, such as ezetimibe or PCSK9 inhibitors.
Figure A1. Lipid metabolism and the atherosclerotic process: How drugs and bioactive compounds can modulate disease progression. Detailed figure description is available on page 19.
Figure A2: Oxidative targets of LDL particle.

Main oxidative targets of LDL are polyunsaturated fatty acids, mostly arachidonic acid (ARA) and linoleic acid (LNA), from phospholipids (PhL) and triacylglycerols (TAG). The first oxidation products are peroxides (LOOH), that may suffer further oxidation and rearrangement resulting in aldehydes and carboxy derivatives. These aldehydes form adducts with lysine residues of apoB, which alters biological properties of LDL. Tyrosine residues can also be directly oxidized by hypochlorous acid (HOCl) generated by myeloperoxidases. Oxidation of LDL also yields phospholipid products as lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA). Another oxidation target is cholesterol molecules, which yields oxysterols products. All products mentioned in the figure have biological effects involved in the atherosclerotic process. Detailed oxidative LDL modifications and biological effects were reviewed by Levitan et al (2010). Abbreviations: ARA, arachidonic acid; CE, cholesterol ester; DHA, docosahexaenoic acid; FA, fatty acid; HETE, hydroxyl-eicosatetraenoic acid; HNE, 4-hydroxy-2-nonenal; HODE, hydroxy-octadecadienoic acid; HPODE, hydroperoxy-octadecadienoic acid; LNA, linoleic acid; LOOH, peroxides; LPA, lysophosphatidic acid; LPC; lysophosphatidylcholine; Lp-PLA2, lipoprotein-associated phospholipase A2; Lys, lysine; MDA, malondialdehyde; oxCholesterol, oxidized cholesterol; oxFA, oxidized fatty acids; oxPhL, oxidized phospholipid; P, phosphate group; TAG, triacylglycerols; Tyr, tyrosine.
HDL ISOLATION PROTOCOL

- Check plasma density: weight 1 mL of plasma in a Beckman Microfuge tube (1.5 ml, REF 357448).
- Bring density to 1.063 with KBr. Calculate the KBr mass (g) using excel sheet.
- Add KBr and mix the sample with the pipette to dissolve the salt.
- Balance the tubes before placing in the rotor (TLA 100.4, Beckman).
- Spin at 44.000 rpm, 18h
- Take the tubes out the rotor really gently. Remove the upper layer (correspond to chylos, VLDL and LDL) with a 1 ml syringe and grey needle (27g1/2).
- Add PBS to all tubes until the 1mL mark. Mix samples and check density again (weight 200 uL of sample in a regular tube and transfer back again).
- Check the total volume of each tube with the pipette (~ 1mL). Use the measured volume and density to calculate KBr mass necessary to bring density to 1.21 (excel sheet).
- Add the salt and mix samples again. Balance the tubes.
- Spin at 49.000 rpm, 22h
- Collect the upper layer (HDL) into a new Beckman tube (1.5mL) with a 1 ml syringe and grey needle (27g1/2). Don’t stir the needle around. Get the very top.
- Washing step. Add KBr solution (1.21d) until the 1.5 mL mark on each tube. Balance the tubes.
- Spin at 49.000 rpm, 22h
- Collect the final upper layer (purified HDL). Use the blue needle (25G1 1/2) with a bent edge (90 degrees). Get the very top. Try not to stir the needle. Leave some behind to avoid albumin contamination.
SECRETARIA DE ESTADO DA SAÚDE  
Coordenadoria de Serviços de Saúde  
INSTITUTO DANTE PAZZANESSE DE CARDIOLOGIA  
Comitê de Ética em Pesquisa

DATA DA ENTRADA: 11 de março de 2014.
DATA DA AVALIAÇÃO: 18 de março de 2014.
CAAE: 27346114.5.0000.5462
Nº DO PROTOCOLO NO CEP: 4424
(este nº deverá cair nas correspondências referentes a este projeto)
Investigadora Principal: Adriana Bertolami Manfredi
Nº Total de Sujeitos no Brasil: 70
Nº Total de Sujeitos no Centre: 70
Área de Conhecimento: Ciências da Saúde
Área Temática Especial: Não se aplica
Fase: Não se aplica
Duração do Estudo: 45 meses
Apoiador Patrocinador: Não informa
Instituição Proponente: Instituto Dante Pazzanese de Cardiologia
CEP Proponente: Instituto Dante Pazzanese de Cardiologia
Centro Coordenador: Instituto Dante Pazzanese de Cardiologia
Instituição Coparticipante: Laboratório de Desenvolvimento de Alimentos Funcionais (LADAF/FCF/USP)

Projeto de Pesquisa Clínica: “EFEITOS DA SUPLEMENTAÇÃO COM OMEGA 3 E FITOSTEROÍS, ASSOCIADOS AO CONSUMO DE CHÁ VERDE, SOBRE BIOMARCADORES DE RISCO PARA ATEROSCLEROSE”.

Considerações/Comentários: O objetivo do estudo é avaliar os efeitos do consumo diário de cápsulas de óleo de peixe rico em EPA+DHA, chocolate contendo fitosteroídes e chá verde sobre biomarcadores de inflamação, hiperlipidemia e estresse oxidativo. Após essa avaliação inicial, os fármacos hiperlipidêmicos serão parcialmente reduzidos, e os mesmos biomarcadores serão novamente avaliados.

Ao se proceder à análise ao projeto em questão, considera-se que:

a) O projeto preenche os requisitos fundamentais da Resolução CNS 466 de 12 de Dezembro de 2012, sobre as Diretrizes e Normas Regulamentadoras de Pesquisa Envolvendo Seres Humanos, do Conselho Nacional de Saúde / Agência Nacional de Vigilância Sanitária e as Boas Práticas de Pesquisa Clínica doICH-GCP.

b) O Comitê de Ética em Pesquisa avaliou e aprovou o Projeto de Pesquisa e o Termo de Consentimento Livre e Esclarecido.

c) O Comitê de Ética em Pesquisa segue os preceitos da Resolução CNS 466 de 12 de Dezembro de 2012, sobre as Diretrizes e Normas Regulamentadoras de Pesquisa Envolvendo Seres Humanos, do Conselho Nacional de Saúde / Conselho Nacional de Ética em Pesquisa / Agência Nacional de Vigilância Sanitária e as Boas Práticas de Pesquisa Clínica do ICH-GCP.

Diante do exposto, O Comitê de Ética em Pesquisa, manifes ta-se pela:

- Projeto de Pesquisa Clínica – Aprovado
- CardioAid-S Plant Sterol Esters – Entregue
- Source OilTM – Entregue

Av. Dr. Dante Pazzanese, 500 • Itaquera • São Paulo – SP • CEP: 04912-009 • Fone: (11) 5565 8040 • E-mail: cep@dantepazzanese.org.br

FORM 901DPF-C Mar/09 rev. 1

1/2
SECRETARIA DE ESTADO DA SAÚDE
Coordenadoria de Serviços de Saúde
INSTITUTO DANTE PAZZANESI DE CARDIOLOGIA
Comitê de Ética em Pesquisa

- Termo de Consentimento Livre e Esclarecido – Aprovação
- Declaração da Pesquisadora: Termo de participação e compromisso do colaborador; Declaração de participação na equipe envolvida; Declaração de realização do estudo (Dr. André Faludi e Dra. Beatriz Cordunus); Declaração de compromisso da instituição em assegurar assistência integral aos participantes da pesquisa; Forma de recrutamento; Termo de compromisso; Declaração de riscos e benefícios, Declaração de infraestrutura; Declaração de propriedade das informações; Declaração de manuseio de material biológico; Publicação dos resultados da pesquisa; Declaração de responsabilidade, direitos e obrigações; Forma de recrutamento; Lista de centros participantes da pesquisa; Declaração de confidencialidade; Declaração de compromisso de notificação de EAS ao CEP; Declaração de participação da equipe envolvida; Declaração sobre currículo Lattes – Entregue
- Carta de subscrição ao CEP – Entregue
- Folha de Rosto – Entregue

O Comitê de Ética em Pesquisa solicita:

a) Informar imediatamente relatório sobre qualquer evento adverso ocorrido.

b) Comunicar qualquer alteração no projeto e no TCLE.

c) Elaborar e apresentar ao CEP relatório semestral sobre o andamento da pesquisa.

Situação: Protocolo Aprovado por este Comitê de Ética em Pesquisa em Reunião Ordinária realizada no dia 18 de Março de 2014.

Pedro Silvio Farsky
Coordenador CEP
CRM 55073

Dr. Pedro Silvio Farsky
Coordenador do CEP
Instituto Dante Pazzanese de Cardiologia

Av. Dr. Dante Pazzanese, 500 • Ibirapuera • São Paulo – SP • CEP: 04012-009 • Fone: (11) 5085 6640 • E-mail: cep@dantepazzanese.org.br

São Paulo, 18 de março de 2014.
ACADEMIC ACHIEVEMENTS

Academic Award:

Best Oral Presentation award (Doctoral category). XX Pharmaceutical Science and Technology Meeting of The Faculty of Pharmaceutical Sciences, University of Sao Paulo. 2015

Publications:

Scolaro, Bianca; Nogueira, Marina; Paiva, Aline; Bertolami, Adriana; Castro, Inar Alves. Effect of nutraceuticals combined with statins on LDL cholesterol depends on the sterol synthesizer/absorber profile of the patients. In: Annual Scientific Session - American College of Cardiology, 2017, Washington. Journal of the American College of Cardiology (Supplement), 2017. v. 69. p. 1704.

Scolaro, Bianca; Soo Jin Kim, Hellen; Castro, Inar Alves. Bioactive compounds as an alternative for drug co-therapy: overcoming challenges in cardiovascular disease prevention. Critical Reviews in Food Science and Nutrition, 2016. DOI: 10.1080/10408398.2016.1235546

Co-authorship

Heffron, Sean; Lin, BingXue; Parikh, Manish; Scolaro, Bianca; Adelman, Steven; Berger, Jeffrey; Fisher, Edward. Changes in HDL Cholesterol Efflux Capacity following Bariatric Surgery are Procedure Dependent. Manuscript acceptable for publication at “Arteriosclerosis, Thrombosis, and Vascular Biology (ATVB)” pending minor revision.

Comunian, Talita A.; Nogueira, Marina; Scolaro, Bianca; Thomazini, Marcelo; Ferro-Furtado, Roselayne; Pallone, Eirira M.; Castro, Inar A. Enhancing stability of omega-3 fatty acids and beta-sitosterol by their coencapsulation using different combinations of wall materials and crosslinkers. Manuscript currently under revision at “Food Chemistry”.

INTERNATIONAL SCIENTIFIC MEETINGS ATTENDED

ACC.17 - 66th Annual Scientific Session - American College of Cardiology (poster presentation). March 17-19 (2017) Washington, DC.

ATVB|PVD 2017. Arteriosclerosis, Thrombosis and Vascular Biology-Peripheral Vascular Disease, 2017 Scientific Sessions (followed by the HDL Workshop). May 04-06 (2017) Minneapolis.
Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9132 - 57574658/1 - Bianca Scolaro
Email: biancascolaro@usp.br
Data de Nascimento: 07/09/1987
Cédula de identidade: RG - 3.334.882-0 - SC
Local de Nascimento: Estado de Santa Catarina
Nacionalidade: Brasileira
Graduação: Nutricionista - Fundação Universidade Regional de Blumenau - Santa Catarina - Brasil - 2010
Mestrado: Mestre em Ciência e Tecnologia de Alimentos (1) - Universidade Federal do Pará - Pará - Brasil - 2013

Curso: Doutorado
Programa: Ciência dos Alimentos
Área: Nutrição Experimental
Data de Matrícula: 10/10/2013
Início da Contagem de Prazo: 10/10/2013
Data Limite para o Depósito: 10/10/2017
Orientador: Prof(a). Dr(a). Inar Alves de Castro - 10/10/2013 até o presente. Email: inar@usp.br
Proficiência em Linguas: Inglês. Aprovado em 10/10/2013
Data de Aprovação no Exame de Qualificação: Aprovado em 09/12/2015
Estágio no Exterior: New York University, Estados Unidos da América - Período de 20/05/2016 até 19/05/2017

Data do Depósito do Trabalho:
Título do Trabalho:
Data Máxima para Aprovação da Banca:
Data de Aprovação da Banca:
Data Máxima para Defesa:
Data da Defesa:
Resultado da Defesa:
Histórico de Ocorrências: Primeira Matrícula em 10/10/2013

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 17/07/2017
Impresso em: 05/09/2017 11:12:12
### 9132 - 8757468/1 - Bianca Scolarol

| Sigla | Nome da Disciplina                                                                 | Inicio | Término | Carga Horária | Cred. | Freq. | Conc. | Exc. | Situação |
|-------|------------------------------------------------------------------------------------|--------|---------|---------------|-------|-------|-------|------|----------|
| BMBS04-3/1 | O Órgão Adiposo como Centro Regulador do Metabolismo (Instituto de Ciências Biomédicas - Universidade de São Paulo) | 14/10/2013 | 25/11/2013 | 30 | 4 | 93 | A | N | Concluída |
| MCM575-5/5 | Aspectos Atuais do Metabolismo de Lipídeos (Faculdade de Medicina - Universidade de São Paulo) | 04/11/2013 | 08/12/2013 | 30 | 4 | 100 | A | N | Concluída |
| FBC5703-2/2 | Análise de Dados Aplicados às Pesquisas Biológicas | 10/03/2014 | 20/04/2014 | 30 | 6 | 100 | A | N | Concluída |
| QEO602-4/6 | Metodologias em Bioquímica e Biologia Molecular: Conceitos e Aplicações (Instituto de Química - Universidade de São Paulo) | 11/03/2014 | 21/04/2014 | 30 | 4 | 100 | A | N | Concluída |
| EDM5761-7/1 | Metodologia do Ensino Superior (Faculdade de Educação - Universidade de São Paulo) | 10/03/2015 | 20/04/2015 | 30 | 0 | - | - | N | Pre-matrícula indeferida |
| BMF5864-2/2 | Fisiopatologia das Espécies Reativas de Oxigênio (Instituto de Ciências Biomédicas - Universidade de São Paulo) | 09/04/2015 | 29/04/2015 | 30 | 4 | 100 | A | N | Concluída |
| FBC5703-6/2 | Ateroescлерose: Fisiopatologia, Diagnóstico e Terapêutica | 03/11/2015 | 30/11/2015 | 30 | 4 | 100 | A | N | Concluída |

| Créditos mínimos exigidos | Créditos obtidos |
|---------------------------|------------------|
| Disciplinas:              | Para exame de qualificação | Para depósito de tese | |
| 0                          | 20                | 26                  |
| Estágios:                 |                   |                     |
| Total:                    | 0                 | 26                  |

Créditos Atribuídos à Tese: 167

**Observações:**

1) Curso com validade nacional, de acordo com o disposto na Portaria MEC nº 1.077, de 31.08.2012.

**Conceito a partir de 02/01/1987:**

- A - Excelente, com direito a crédito;
- B - Bom, com direito a crédito;
- C - Regular, com direito a crédito;
- R - Reprovado;
- T - Transferência.

Um (1) crédito equivale a 15 horas de atividade programada.

**Última ocorrência:** Matrícula de Acompanhamento em 17/07/2017

**Impresso em:** 05/09/2017 11:12:12