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Ponta Delgada, 2017

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Supervisor: Maria Leonor Pereira de Almeida Pavão Sequeira de Medeiros

Co-supervisor: José António Bettencourt Baptista

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Declaração

Este é um trabalho original, elaborado especificamente para dar cumprimento ao exigido para obtenção do grau de Doutor em Biologia.

Para os devidos efeitos, declaro que o mesmo, na sua totalidade, ou nas suas partes, não foi submetido a qualquer editora, nem está publicado.

Ana Patrícia Rego Lima

Julho de 2017

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To my family,

namely my son

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

Sir William Bragg

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Acknowledgments

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Preface

Atherosclerotic cardiovascular diseases (CVD) are the largest cause of morbidity and premature death worldwide and are estimated to cost the EU economy almost €196 billion a year, of which €60 billion with coronary artery disease (Nichols *et al*., 2012). In Portugal, The Azores archipelago has the highest standardized mortality rate for coronary artery disease (CAD), which constitutes a huge public health concern. Finding its causes is not only urgent but also a challenging target.

Since classic risk factors do not explain all CVD cases and symptoms generally arise in advanced stages of atherosclerosis, there is a need to find precocious biomarkers for this pathology. Low molecular weight aminothiols, like homocysteine, cysteine, cysteinylglycine and glutathione, are important intra and extracellular redox buffers metabolically interrelated, whose altered profile is an indirect marker of oxidative stress and vascular injury. Over the last years, they have been seen with mounting interest, since oxidative stress is a unifying mechanism for many classic CVD risk factors and is also linked to pro-inflammatory processes, a usual feature in atherosclerosis and related diseases.

Since 1969 that hyperhomocysteinemia is a well-recognized independent risk factor for CVD. However, clinical trials with B-vitamins for lowering plasma homocysteine levels have not succeeded in reducing CVD risk, which suggests that hyperhomocysteinemia is associated with some other causal mechanism. On the contrary, hypercysteinemia, which is strongly correlated with hyperhomocysteinemia, only recently has been hypothesized to play a causal role in obesity and other metabolic disorders.

This doctoral thesis follows the same theme I have worked in to obtain my master degree at the University of Azores in 2011.

This dissertation is organized in five chapters. Chapter 1, named General Introduction, begins with a summarized review of the current literature on atherosclerosis and its conventional and emerging risk factors, namely related to oxidative stress. A special attention is dedicated to the metabolism of aminothiols. It also includes the definition of the objectives and a brief demographic characterization of the study groups further detailed in the following chapters.

Chapters 2 to 4 are in a format that is in line with the concerns supporting the definition of the objectives of this thesis, thus facilitating a comprehensive perception of the whole research work and its results. This organization also represents a first approach for the future elaboration of scientific papers intended to submit for publication after the presentation and public discussion of this thesis.

Chapter 2 regards the evaluation, for the first time, of plasma aminothiol profile (and its major determinants) in two groups of apparently healthy subjects from Ponta Delgada and Lisbon, two urban cities in Azores and mainland Portugal, respectively. Classic CVD risk factors are also considered.

Chapter 3 presents both plasma and erythrocyte aminothiol profile in 174 consecutive azorean subjects undergoing elective coronary angiography, in order to find biochemical predictors for CAD and its severity in high-risk subjects under preventive medication. The major aminothiol determinants and classic CVD risk factors are also examined.

Chapter 4 essentially brings together some circulating redox parameters, namely antioxidant vitamins and copper levels, in the same group of subjects who performed coronary angiography, also aiming the finding of predictors for CAD and its severity in The Azores archipelago.

Finally, Chapter 5 crosslinks and summarizes the main conclusions of this work, its limitations and presents suggestions for future research.

Abstract

In Azores, the standardized mortality rate for coronary artery disease (CAD) is near twice the observed in mainland Portugal. Along with the traditional ones, other risk factors have to be searched to clarify the pathophysiology of atherosclerosis-related disorders and therefore the above related differences between the two Portuguese regions.

Aminothiols, such as homocysteine (Hcy), cysteine (Cys), cysteinylglycine (Cys-Gly) and glutathione (GSH), when in altered concentrations, are an indirect marker of oxidative stress, which plays a central role in the genesis and progression of atherosclerosis. They are actually being viewed with high interest in CAD prediction.

The prevalence of classic risk factors and plasma aminothiol profile were evaluated in healthy subjects from Ponta Delgada (PDL) and Lisbon cities, aiming to find precocious biomarker(s) of the disease. The concentrations of aminothiols in both plasma and erythrocyte, as well as several redox parameters were also determined in 174 angiographically documented CAD and non-CAD azorean subjects under medication, searching for biochemical predictors of CAD and its severity.

In the cross-sectional study, no differences in prevalence of conventional CAD risk factors were found among healthy subjects, except in central obesity and related physical inactivity, which were both higher in PDL men than in those from Lisbon. Hypercysteinemia, suggested to result from high sulfur-rich amino acids diets and/or vitamin B_{12} malabsorption, revealed to be significantly more prevalent in PDL vs. Lisbon subjects (18% vs. 4%, *P*=0.001), namely in male gender. Moreover, plasma Cys levels predicted waist circumference (β coefficient = 0.102, *P*=0.032) and concomitant central obesity and were also associated with insulin resistance. Nevertheless, hyperhomocysteinemia prevalence was similar in both groups, despite PDL subjects exhibited a higher rate of vitamin B_{12} deficiency than those from Lisbon (19% vs. 6%, *P*=0.003).

In the case-control study, about three quarters of CAD patients were male. Multivariate logistic regression analysis showed that hypercysteinemia (≥ 286μM - 4th quartile) was an independent predictor for CAD in high-risk subjects under medication in either primary or secondary prevention, with a 3-fold higher risk of disease (OR [95% CI] = 2.97 [1.34-6.59], *P*=0.007). Again, hyperhomocysteinemia was not a relevant risk factor for CAD in Azores. Plasma copper levels, which were strongly associated with acute inflammation, showed to be a CAD predictor in primary but not in secondary prevention.

Owing to the nature of both studies, a cause-effect relationship between high plasma Cys levels and central obesity or CAD risk could not be derived, but results strongly suggest that hypercysteinemia is a potential risk factor for metabolic disorders and CAD in The Azores archipelago, a hypothesis that claims for confirmation through future large prospective studies.

Keywords: coronary artery disease, aminothiols, hypercysteinemia, central obesity, Azores

Resumo

Nos Açores, a taxa de mortalidade padronizada por doença arterial coronária (CAD) é cerca do dobro da de Portugal continental. Para além dos factores de risco tradicionais, impõe-se a investigação de outros factores, no sentido de clarificar a fisiopatologia das doenças ateroscleróticas e, consequentemente, as diferenças acima referidas entre as duas regiões portuguesas.

Os aminotióis, como a homocisteína (Hcy), a cisteína (Cys), a cisteinil-glicina (Cys-Gly) e o glutationo (GSH), quando presentes em concentrações alteradas, são marcadores indirectos de stresse oxidativo, o qual, por sua vez, desempenha um papel fundamental na génese e progressão da aterosclerose. São por isso encarados com muito interesse na predição de CAD.

Avaliou-se a prevalência dos factores de risco tradicionais, bem como o perfil aminotiólico plasmático em indivíduos saudáveis das cidades de Ponta Delgada (PDL) e de Lisboa, com o objectivo de encontrar biomarcador(es) precoce(s) da doença. Foram também determinadas as concentrações dos aminotióis no plasma e no eritrócito, assim como vários parâmetros redox, em 174 indivíduos açorianos de alto risco (e preventivamente medicados) com e sem CAD, tal como documentado por angiografia coronária, a fim de se encontrar preditores bioquímicos de CAD e da sua severidade.

No estudo transversal que envolve indivíduos saudáveis, não se encontraram diferenças entre os grupos de estudo (PDL vs. Lisboa) relativamente à prevalência dos factores de risco convencionais, com a excepção da obesidade central e da inactividade física, ambas mais prevalentes nos homens de PDL. A hipercisteinémia (que se sugere resultar de dietas ricas em aminoácidos com enxofre e/ou de uma deficiente absorção da vitamina B_{12}) revelou ser significativamente mais prevalente nos indivíduos de PDL vs. Lisboa (18% vs. 4%, *P*=0,001), nomeadamente no sexo masculino. Além disso, os níveis plasmáticos de Cys mostraram

predizer os do perímetro abdominal (coeficiente β = 0,102, *P*=0,032) e a inerente obesidade central, estando também associados a resistência à insulina. Contudo, a prevalência da hiper-homocisteinémia foi semelhante nos dois grupos de estudo, apesar de a frequência de deficiência em vitamina B_{12} ter sido maior nos indivíduos de PDL do que nos de Lisboa (19% vs. 6%, *P*=0,003).

No estudo caso-controlo, cerca de três-quartos dos doentes com CAD eram homens. Na análise de regressão multivariada, a hipercisteinémia (≥ 286μM – 4º quartil) revelou ser um preditor independente de CAD (ao nível das prevenções primária e secundária) em indivíduos de alto risco referenciados para angiografia coronária, correspondente a um risco 3 vezes superior de contrair a doença (OR [95% IC] = 2,97 [1,34-6,59], *P*=0,007). Uma vez mais, a hiper-homocisteinémia não evidenciou ser um factor de risco relevante de CAD no arquipélago dos Açores. O nível plasmático de cobre, que se mostrou fortemente associado à fase aguda da inflamação, também evidenciou capacidade preditiva de CAD na prevenção primária, mas não na prevenção secundária.

Dada a natureza de ambos os estudos, não foi possível estabelecer uma relação directa de causa-efeito entre os níveis plasmáticos de Cys e a obesidade central, ou o risco de CAD. Contudo, os resultados encontrados sugerem fortemente que a hipercisteinémia é um factor de risco potencial para as doenças metabólicas e para a CAD no arquipélago dos Açores, uma hipótese que urge ser confirmada em futuros estudos prospectivos de maior dimensão.

Palavras-chave: doença arterial coronária, aminotióis, hipercisteinémia, obesidade central, Açores

List of abbreviations

AGEs – advanced glycation end-products

BHMT – betaine homocysteine methyltransferase

BMI – body mass index

BP – blood pressure

CAD – coronary artery disease

CBS – cystathionine β-synthase

CRP – C-reactive protein

CVD – cardiovascular diseases

Cys – cysteine (without specification of its form)

CySS – cystine (oxidized cysteine)

Cys-Gly – cysteinylglycine (without specification of its form)

DM2 – type 2 diabetes mellitus

eGFR – estimated glomerular filtration rate

Hcy – homocysteine (without specification of its form)

HcySS – homocystine (oxidized homocysteine)

Hcys – hypercysteinemia

HDL – high density lipoprotein

HDL-c – high density lipoprotein cholesterol

HHcy – hyperhomocysteinemia

HOMA-IR – homeostasis model assessment of insulin resistance

HPLC – high performance/pressure liquid chromatography

GGT – γ-glutamyl transferase or γ-glutamyl transpeptidase

GCS/GCL – γ-glutamyl-cysteine synthetase or γ-glutamyl-cysteine ligase

Glu – glutamate

Gly – glycine

GPX – glutathione peroxidase

GR – glutathione reductase

GSH – glutathione (without specification of its form)

rGSH – reduced glutathione

GSSG – glutathione disulfide or oxidized glutathione

LDL – low density lipoprotein

LDL-c – low density lipoprotein cholesterol

MDA – malondialdehyde

Met – methionine

Mrp/Abcc – multidrug-resistance-related protein, a subfamily of the ATP-binding cassette (ABC) transporters

MS – methionine synthase

MTHFR – 5,10-methylene tetrahydrofolate reductase

NADPH – nicotinamide adenine dinucleotide phosphate

NCEP/ATP III – national cholesterol education program/adult treatment panel III

NF-kB – nuclear factor- kappa B

PDL – Ponta Delgada

PLP – pyridoxal 5'-phosphate

RBC – red blood cells

ROS – reactive oxygen species

SAH – S-adenosylhomocysteine

SAM – S-adenosylmethionine

SOD – superoxide dismutase

TC – total cholesterol

TG – triglycerides

THF – tetrahydrofolate

5-methylTHF – 5-methyltetrahydrofolate