Proteomics in cardiovascular diseases: unveiling sex and gender differences in the era of precision medicine

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Keywords:

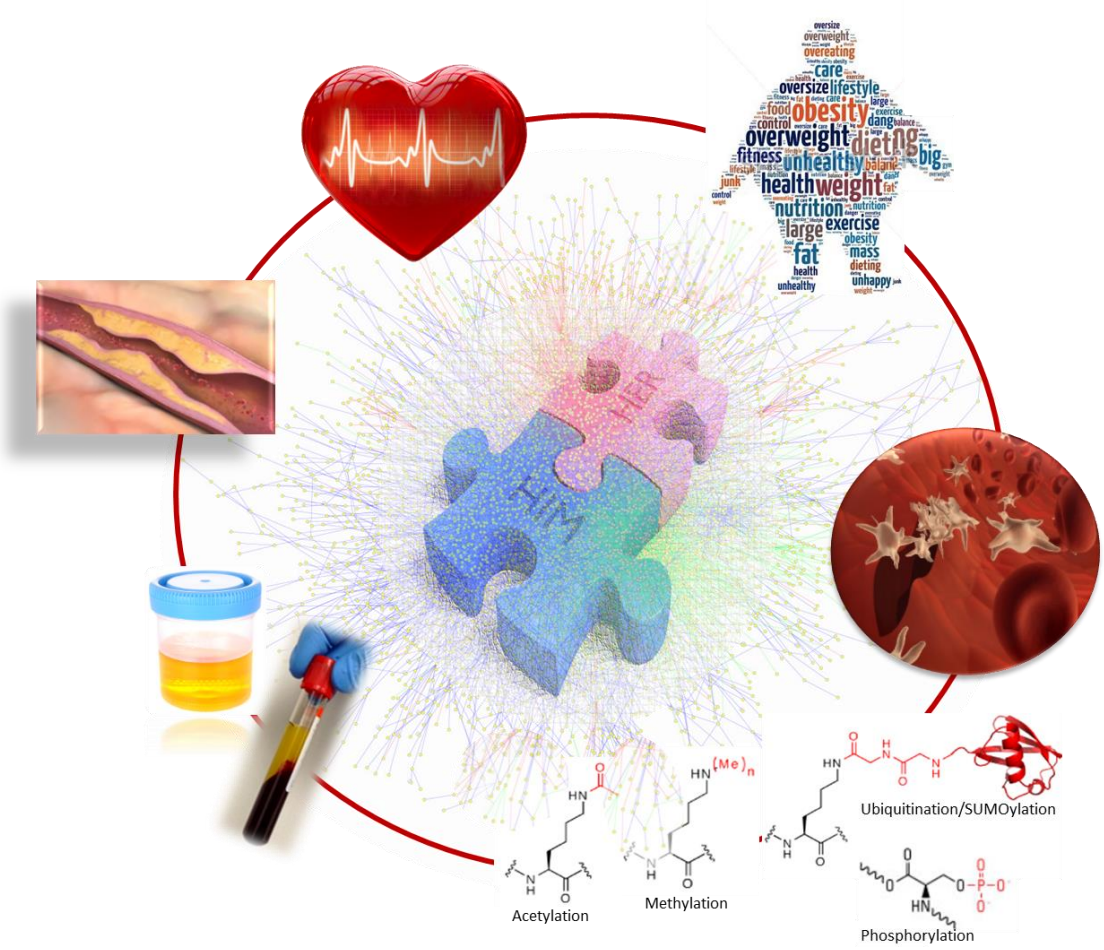
Sex differences; Gender; Cardiovascular disease; Risk Factors; Oxidative stress; Proteomics.

In Press, Accepted Manuscript. https://doi.org/10.1016/j.jprot.2017.11.012

ABSTRACT

Cardiovascular diseases (CVDs) represent the most important cause of mortality in women and in men. Contrary to the long-standing notion that the effects of the major risk factors on CVD outcomes are the same in both sexes, recent evidence recognizes new, potentially independent, sex/gender-related risk factors for CVDs, and sex/gender-differences in the clinical presentation of CVDs have been demonstrated. Furthermore, some therapeutic options may not be equally effective and safe in men and women. In this context, proteomics offers an extremely useful and versatile analytical platform for biomedical researches that expand from the screening of early diagnostic and prognostic biomarkers to the investigation of the molecular mechanisms underlying CDVs. In this review, we summarized the current applications of proteomics in the cardiovascular field, with emphasis on sex and gender-related differences in CVDs.

Graphical abstract



In Press, Accepted Manuscript. https://doi.org/10.1016/j.jprot.2017.11.012

1. Introduction

Cardiovascular diseases (CVDs) are the world's leading cause of morbidity and mortality, accounting for more than 17 million deaths annually [1], and cause immense health and economic burdens [2, 3]. In line with the recommendations of the World Health Organization (WHO), the principal health organizations in the field of heart diseases and stroke (such as the American Heart Association and the European Society of Cardiology), formulated recommendations to drive organizational priorities and guide actions to prevent CVDs in clinical practice [4, 5]. In accordance with the strategic view of these recommendations, to achieve the goal of significantly reducing deaths attributable to CVDs continued emphasis is needed on the treatment and control of health behaviors and risk factors at both the population and the individual level [2, 5]. In the era of precision medicine, the key challenge is to bridge the gaps in our knowledge about sex- and gender-related differences in the pathophysiology of the cardiovascular system, since increasing evidence supports the notion that an individual's sex is one of the most important modulators of disease risk and response to treatment [6-8].

Indeed, a large amount of correlative data unveils the existence of sexual diversities in human physiology and differential susceptibility to a wide variety of pathologies including CVDs [9, 10]. Beyond environmental and social differences between men and women (e.g., occupational hazards, lifestyle, social stresses, access to healthcare) that can contribute to gender differences in CVDs, sex hormones have long been found to account for some sex-related differences in CVDs, and some molecular mechanisms mediating these effects have recently been elucidated [8, 10, 11]. Moreover, sex chromosomes are beginning to be recognized as important determinants of sexual dimorphism in the development of CVDs, independent of sex hormones [8, 10-12]. In this Review, we consider the evidence for sex and gender differences in CVDs and summarise the proteomic research that has been conducted in this field.

2. Sex-specific and gender-specific cardiovascular research

CVDs have long been considered as male diseases, an assumption that stems largely from observations that CVDs in women develop later in life than in men, and the misperception that CVDs among women are not

In Press, Accepted Manuscript. https://doi.org/10.1016/j.jprot.2017.11.012

as severe as they are in men [13]. In line with this view, until recently cardiovascular research was predominantly conducted in men and it was assumed that clinical approaches based on research findings involving men were equally relevant for women [13]. However, a growing body of evidence has progressively revealed the importance of CVDs in women and has fostered the awareness of sex- and gender-related differences in the occurrence, management and outcomes of CVDs [13]. Marked progress has been made in the involvement of women in large-scale population studies and clinical trials. Nevertheless, several gaps in our understanding of sex- and gender-related diversities in cardiovascular health still persist. Moreover, the use of female animals, cells, or tissues, and sex-based reporting in preclinical investigations have not been equally implemented [14], in spite of the publication of a planned policy from the U.S. National Institutes of Health (NIH) to balance sex in cell and animal studies [15]. In this regard, it is important to highlight the value of preclinical studies for understanding the molecular bases of sex differences, since such studies: 1) enable scientists to take full advantage of the power of molecular genetics and 'omics technologies; 2) allow the control of variables such as diet, environment, exercise; and 3) offer the opportunity to quantify the extent of sex or gender contribution to the biological outcome, since in experimental animals gender has limited impact [9].

3. Sex and gender differences in CVD risk factors

Most of the traditional risk factors for CVDs, including elevated blood pressure, dyslipidemia, excess body weight and obesity, diabetes, and cigarette smoking, are similar between men and women, but for some of them the impact differs between the sexes; furthermore, recent evidence has emerged that recognizes new, potentially independent female-specific risk factors (Figure 1) [8, 16].

3.1 Major risk factors affecting both men and women

3.1.1 Elevated systolic blood pressure

Elevated systolic blood pressure (SBP) is one of the leading risk factors for global mortality and for CVDs. In 2015, the prevalence of raised blood pressure was around 20% in females aged 18 and over and around

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24% in males [17]. Studies have reported conflicting results on whether the association between increments in SBP and CVDs differs between sexes [16]. A pooled analysis carried out in 2013, including data from prospective cohort studies on more than 1.2 million individuals and over 50,000 cardiovascular events, found that every 10 mmHg increment in SBP was associated with a 15% increased risk of coronary heart disease (CHD) and a 25% increased risk of stroke in both men and women, indicating a similar impact of hypertension on cardiovascular outcomes in both sexes [18]. In contrast, results of a recent meta-regression analysis of US population-based studies indicate that women experienced a 10% greater risk in CVDs per 10 mmHg increment in SBP than men, after adjusting for age and baseline SBP [19].

3.1.2 Dyslipidemia

Raised total cholesterol (TC) is estimated to account for over 2.6 million deaths (4.5% of total) worldwide every year [20]. The prevalence of elevated TC is similar in men and women [20] and studies addressing the possible sex/gender-specific effects of TC on CVD risk have reported inconsistent results [21]. The first systematic meta-analysis evaluating the impact of TC on CVD risk in women compared with men included data from over one million individuals and more than 20,000 CHD and 16,000 stroke events [21]. This analysis found that for every 1-mmol/L increment in TC, the risk of CHD increased by 20% in women and by 24% in men, indicating essentially a similar TC-related risk of CHD in both sexes [21].

In population studies, high-density lipoprotein cholesterol (HDL-C) is inversely related to the risk of myocardial infarction and death [22]. Low HDL was initially suggested to be more predictive of coronary risk in women compared to men [23]; however, analyses of more than 300,000 people from 68 long-term prospective studies contributing to the Emerging Risk Factors Collaboration (ERFC) analysis on the associations of major lipids and apolipoproteins with the risk of vascular disease indicated that, after adjustment for other cardiovascular risk factors, the association between HDL cholesterol levels and fatal CHD did not vary significantly by sex: each 1-SD increase in HDL-C lowered the risk of CHD mortality by 26% in women and by 21% in men [24].

3.1.3 Diabetes mellitus

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Diabetes mellitus (DM) is an important predictor of a person's risk of vascular disease [25]. It is one of the largest global health emergencies of the 21st century, with an estimated global prevalence of over 400 million and a projected increase to 642 million by 2040, which poses an enormous burden on healthcare [26]. Although there is little gender difference in the global number of people with diabetes [26], compelling evidence indicates that women, compared to men, have a significant and clinically important higher excess risk of both CHD and stroke consequent to diabetes (44% and 27%, respectively) [27-29]. Furthermore, diabetic women have a higher risk of developing heart failure (HF) or peripheral arterial disease (PAD) compared with diabetic men [30]. Several hypotheses have been proposed to explain how diabetes confers a female disadvantage in terms of vascular risk, but the exact mechanisms remain unclear [16, 31]. An attractive – but still unproven – hypothesis is that women live in a suboptimal glycemic ('prediabetic') state for a longer period of time than men, during which their metabolic profile continues to deteriorate relative to men, so that considerable vascular damage has already occurred by the time they are clinically diagnosed with diabetes [31].

3.1.4 Body fat, excess body weight and obesity

Excess body weight is another major risk factor for CVDs and currently one of the greatest public health issues worldwide [1]. According to the WHO global estimates, excess body weight has reached epidemic proportions globally: in 2014, more than 1.9 billion adults were overweight (38% of men and 40% of women); of these, over 600 million were obese [32]. The association between body mass index (BMI) and CHD has been shown to be the same between men and women in the large-scale analyses of the Prospective Studies Collaboration [33] and the ERFC [34], and in a meta-analysis including data from 95 cohorts with more than 1.2 million participants [35]. These results indicate that increased BMI has the same deleterious effects on the risk of CHD in women and men. However, there are numerous differences between males and females regarding body fat, excess body weight and obesity that could be due to either direct activation by sex steroids or by sex steroid-independent mechanisms. Although men generally have greater body weight than women, the proportion of body weight as fat is greater in women and there is a clear hormone-related sexual dimorphism in the patterns of body fat storage and fat metabolism [36]. In

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their fertile age, females store the lipids in excess in subcutaneous deposits (such as the gluteal femoral region) that are believed to be associated with lower cardiometabolic risk than the abdominal (visceral) fat accumulation that predominates in men [36]. When ovarian activity ceases with the onset of menopause, this female advantage is lost and women become more vulnerable to the risks of an obesogenic environment [9]. In addition, the concept has recently emerged that the complex and different effects of obesity on CVDs could in different cases be detrimental, or innocuous, or even protective [37]. Indeed, the possible existence of a metabolically healthy obese phenotype (more appropriately defined as a lower risk form of obesity), the important role of regional body fat distribution and ectopic fat accumulation, and the presence of an "obesity paradox" in patients with CVDs, are all observations which emphasize the remarkable heterogeneity of obesity [37]. Thus, given the complex metabolic roles of the adipose tissue [38] and the importance of obesity as a driver of several major CVD risk factors [31], more research is needed on gender-specific pathophysiology of obesity development.

3.1.5 Cigarette smoking

Smoking (including second-hand smoking) is an established cause of a myriad of diseases and according to the WHO it is currently responsible of more than 7 million deaths across the world each year [39]. With regard to CVDs, it is well known that smoking negatively affects endothelial function, oxidative processes, platelet function, fibrinolysis, inflammation, and vasomotor function, thus promoting the development of both atherosclerosis and the superimposed thrombotic complications [5]. According to these proatherogenic roles of cigarette smoking, the 10-year risk of fatal events is approximately doubled in smokers compared to non-smokers [5]. While the beneficial effects of smoking cessation on coronary risk are similar in women and men, the mortality from CVDs is higher in female than male smokers, even after adjustment for other risk factors [40]. Furthermore, a meta-analysis of data from 75 prospective cohort studies and nearly 2.4 million subjects showed that female smokers had a 25% higher risk of developing CHD than men with the same exposure to tobacco smoke [41]. With regard to stroke, a second meta-analysis, involving data from 81 cohorts worldwide and nearly 4 million individuals did not find an overall greater excess risk of smoking in women compared with men, but it found a 10% higher risk in female

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smokers in Western populations, where smoking is a long-standing habit also among women [42]. The molecular basis underlying such differential female susceptibility to tobacco smoke is not currently understood. Interestingly, a recent report on genome-wide profiling in white blood cells found that the expression or methylation of several genes with a key role in the pathogenesis of CVDs (especially genes involved in thrombin signaling) is altered by smoking significantly more in females than in males [43]. These results underline the potential of blood-based omics profiling in sex/gender-specific risk assessment.

3.2 Women-specific risk factors

The unique aspects of cardiovascular health in women have been comprehensively reviewed by Garcia and colleagues [30], who provided an in-depth analysis on sex and gender differences related to clinical practice in the prevention, diagnosis, and treatment of CVDs. Sex steroid hormones, especially estrogen (the major sex steroid in females), have a plethora of physiological effects on the cardiovascular system, as well as indirect effects mediated through changes in metabolism and coagulation [8, 11, 44]. In view of that, it is thought that changes in circulating levels of endogenous sex hormones, such as those that occur in women during pregnancy and menopause, can affect current and future CVD risk [8]. In addition, exogenous hormones in the form of hormonal contraceptives and menopausal hormone therapy modulate the hormonal environment, and subsequently women-specific CVD risk [8]. The latter topics are not addressed in this review but have been covered previously [8, 45-47].

3.2.1 Hypertensive disorders of pregnancy and gestational diabetes mellitus.

The vascular, metabolic and immunological adaptations that occur to a woman’s body during pregnancy pose a substantial challenge to the cardiovascular system, and pregnancy-associated disorders are often the result of the mother's inability to adapt to this vascular and metabolic stress [8, 16]. Accordingly, complications such as hypertensive disorders (including the conditions of gestational hypertension and preeclampsia) or gestational DM represent important women-specific factors to consider in risk assessment, since they can place a woman at long-term risk of developing CVDs, or reveal a preexistent cardiovascular dysfunction [8, 16]. Preeclampsia (defined as pregnancy-related hypertension accompanied

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by proteinuria) occurs in 1–2% of all pregnancies [5]. A meta-analysis by Bellamy et al. [48] found that in comparison to women with normal pregnancies, women who suffered from preeclampsia had a greater relative risk for developing hypertension, CHD, and stroke later in life. Gestational hypertension affects 10–15% of all pregnancies [5]. The associated risk of later CVDs is lower than for preeclampsia, but is still elevated [49]. Gestational DM has a prevalence of 3-5% of all pregnancy and is similarly associated with an increased risk of future CVDs. Most of this risk appears to be mediated by a sharply elevated likelihood of future type 2 DM in women with gestational DM compared to women with normoglycaemic pregnancies, with up to 50% developing type 2 DM within 5 years [50]. Interestingly, women with a history of hypertensive disease in pregnancy have a higher risk for developing type 2 DM [49] and women with gestational DM have a higher risk for gestational hypertension and preeclampsia [51], suggesting a close link between the vascular and the metabolic complications of pregnancy, which are both associated with increased risk of CVDs later in life.

3.2.2 Menopause

In their fertile age, women are relatively protected against CVDs, compared with age-matched men. However, this sex gap narrows after menopause [30]. The decrease in ovarian activity during and after menopause goes hand in hand with an increased risk of CVDs in women, partially because the deleterious biological changes consequent to the loss of endogenous estrogens favor hypertension, diabetes, hyperlipidemia, central obesity and the metabolic syndrome [8, 16, 52]. Two meta-analyses that assessed the relationship between age at menopause and CVD risk found that menopause before age 50 was associated with a 25% higher risk of CVDs [53], while natural menopause <40 years, better defined as primary ovarian insufficiency, was related to an increased hazard ratio (HR) of CHD of 1.69 and an HR of 1.61 for total CVDs [54]. However, menopausal hormone therapy increases the risks of serious disease, such as breast or endometrial cancers, and its cardiovascular effects are controversial; thus, the current consensus is that it should never be prescribed for the aim of preventing CVDs [8, 30, 47].

3.2.3 Other emerging, non-traditional CVD risk factors in women

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Other conditions that are emerging as non-traditional CVD risk factors in women include preterm delivery, systemic autoimmune diseases (such as rheumatoid arthritis and systemic lupus erythematosus), breast cancer treatments, and depression [16, 30] (Figure 1).

Spontaneous preterm delivery appears to be an independent risk factor for the development of ischemic heart disease (IHD), stroke, and overall CVDs according to a meta-analysis including 10 cohort studies from five north western European countries and follow-ups that ranged from 12–35 years [55]. Using data from ≈70,000 participants in the Nurses' Health Study II, Tanz et al. [56] recently demonstrated that preterm delivery (<37 weeks gestation) was associated with an increased risk of future CVDs (HR of 1.42); remarkably, only a modest proportion of the increased risk was accounted for by the postpartum development of conventional CVD risk factors, which suggests that the association between spontaneous preterm delivery and CVD risk is mediated by alternative mechanisms [56]. At present, these mechanisms are not well understood, but it has been suggested that the increased inflammatory status observed in women with preterm delivery may play a role [55, 56].

Many population studies attest the association between excess cardiovascular burden and systemic autoimmune diseases [57]. Little is known about the relationships between systemic autoimmune diseases and sex, but it has been proposed that the microvasculature may play an important role in the predisposition of women with autoimmune diseases to develop accelerated CVDs [58]. Since systemic autoimmune diseases are generally more prevalent among female subjects, they represent more common CVD risk factors in women compared to men [30, 58].

Breast cancer treatments also represent important causes of excess CVD risk in women, due to incidental exposure of the heart to the deleterious effects of ionizing radiations and to the cardiotoxicity of the breast cancers chemotherapeutic agents [30, 59]. Since there has been an enormous improvement in the survival rates of breast cancer, the focus on cardiac health in breast cancer patients is becoming a priority.

Increasing evidence indicates that depression is a prevalent risk factor for the development of CHD and a predictor of unfavourable outcomes after a CHD event [60]. Overall, hundreds of studies investigated the relationship between depression and the onset and progression of CHD [60]. These investigations suggest

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that, although depression is associated with other cardiac risk factors (such as sedentary life), it is an independent risk factor for CHD morbidity and mortality [60]. Since depressive disorders are more common in female subjects, especially young women, they can affect women's CVD risk disproportionately [61, 62]. Many possible pathogenetic mechanisms have been proposed to explain the relationship between CVDs and depression. Recently, we (C.B.) found an association between the brain-derived neurotrophic factor (BDNF) Val66Met polymorphism and the enhancement of thrombosis in an experimental model of carotid injury or the increased occurrence of acute coronary syndromes in a human coronary artery disease (CAD) cohort [63]. These findings provide a potential mechanistic link between depression and CAD; however, major challenges remain to be addressed for assessing the potential cause–effect relationships of this association in humans. Given the enormous public health impact of depression and heart disease, efforts are needed to gain further insights into gender and individual differences in the susceptibility for depression and CVDs, which could contribute to the improvement of both prevention and treatment.

4. Sex and gender differences in CVD manifestations and underlying pathophysiological mechanisms

CVDs occur and progress differently in the two sexes [10, 11]. An overview of sex and gender differences in the manifestations of CVDs and the underlying pathophysiological mechanisms is provided below.

4.1 Ischemic heart disease

In the context of IHD, evidence of an uniquely female pattern of disease is emerging, including not only atherosclerotic CAD, but also an expanded spectrum of coronary disease, comprising coronary microvascular dysfunction (CMD), spontaneous coronary dissection (SCAD), and Takotsubo cardiomyopathy [30]. Furthermore, women with IHD typically have a poorer prognosis then men [30].

With regard to CAD, women have often a non-obstructive pattern that differs from the traditional male model of obstructive CAD [30]. Moreover, several histological observations attest the existence of differences in the morphology of atherosclerotic plaques underlying CAD in men and women [7]. According to registry data of patients dying from coronary thrombosis, plaque rupture is more frequent in men than

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women, whereas plaque erosion is more frequent in women than men [64, 65]. In women, this pattern seems to be affected by the hormonal status, since pathological observations indicate that the incidence of plaque rupture is higher than that of erosion in postmenopausal compared to premenopausal women (87% vs. 17%, P = 0.001) [65].

CMD, defined as limited coronary flow reserve and coronary endothelial dysfunction, is another frequent cause of IHD in women, associated with increased rate of cardiac death, stroke, or HF. It is characterised by a decrease in the size of epicardial vessels and microvasculature, diffuse atherosclerotic disease, increased arterial stiffness and fibrosis, altered remodeling, and the presence of endothelial or smooth muscle dysfunction [30]. Interestingly, impaired coronary flow reserve in the absence of obstructive CAD has recently been associated with excess cardiovascular risk in women [66].

SCAD is an uncommon cause of acute myocardial ischemia that occurs when a tear forms between the layers of a coronary artery, and most frequently (>90%) affects women below 60 years of age [30, 65]. The classic presentation is of a young healthy woman, without traditional atherosclerotic CVD risk factors, and sudden onset of acute coronary syndrome [30].

Takotsubo cardiomyopathy, affecting postmenopausal women in nearly 90% of the reported cases, is another sex-specific cause of transient acute ischemic heart disease. The etiopathology of Takotsubo cardiomyopathy is not clear; proposed mechanisms include multivessel coronary artery spasm, impaired cardiac microvascular function, endogenous catecholamine-induced myocardial stunning and myocarditis [30, 65].

4.2 Heart failure

HF, which occurs when the heart muscle is weakened and cannot pump enough blood to meet the body's needs for blood and oxygen, has a high prevalence in old age, affecting more than 10% of those above 70 years in Western societies and typically more women than men [11]. In particular, women are ≈twice as likely as men to develop HF with preserved ejection fraction, a condition for which no treatment has yet proved effective [11, 30].

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4.3 Other vascular diseases

Other manifestations of CVD showing sex/gender-related differences include ischemic stroke, peripheral arterial disease (PAD), and abdominal aortic aneurysm (AAA).

Women have an increased lifetime incidence of stroke compared with men, largely because of a sharp increase in stroke risk in older postmenopausal women, and an increased lifetime prevalence of stroke risk factors, including hypertension, abdominal obesity and metabolic syndrome [30]. Furthermore, elderly women have more severe strokes and greater disability compared with age-matched men [67]. With regard to the underlying pathohistological characteristics, evidence suggests that carotid plaque morphology differs between men and women: women with a carotid stenosis have more stable plaques than men, independent of clinical presentation and cardiovascular risk profile [68], while plaques from men are associated with more cellularity, more inflammatory infiltrates, and more neovascularization [69].

PAD is now recognised to be associated with comparable morbidity and mortality to CAD and stroke, and is associated with significantly reduced quality of life [30]. Similar to CAD, PAD is more prevalent in men than women at younger ages, but the incidence rises in women after menopause; in addition, women generally display more severe PAD compared to men and experience greater complications [67].

AAA is a localized ballooning of the abdominal aorta. It is 4 to 6 times more prevalent in men than women, and develops in women ≈10 years later than in men, although it has worse outcomes in women [30]. The underlying reasons for males being predisposed are still not completely clear because of the disease's complex pathogenesis [67].

5. Gender proteomics in CVD

A clear understanding of the mechanisms underlying sexual dimorphisms in pathophysiology is crucial for precision medicine, in which the knowledge of the molecular bases of diseases is considered essential for the definition of appropriate preventive and therapeutic approaches [9]. In this context, proteomics, and 'omics approaches in general, can provide powerful tools to analyze physiological and disease-induced biological states at the molecular level, taking into account both the organism's intrinsic properties, such as

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genetic factors, and the effects of lifestyle, diet, and environment. The development of sophisticated analytic platforms to handle increasingly complex data now enables the analysis of complex biological samples with a high throughput rate, offering an extremely useful and versatile analytical tool for biomedical researches that expand from the screening of early diagnostic and prognostic biomarkers to the investigation of the molecular mechanisms underlying CDVs. Proteomic studies focused on sex and gender-related differences in CVDs are still very rare, but they are expected to increase in the coming years and will provide novel insights into the pathophysiology and clinical manifestations of these diseases.

In the following paragraphs, we illustrate the most relevant examples of proteomics studies to date that have focused on sex/ gender-related differences performed to date in the context of CVDs.

5.1 Proteomics of biological fluids

5.1.1 Plasma and serum

Although proteomics of biological fluids has the potential to identify novel proteins that can improve the accuracy of cardiovascular risk prediction, many challenges still exist. Nowadays the plasma, with more than 10,000 proteins identified (http://www.plasmaproteomedatabase.org), represents the most challenging proteome due to the exceptionally wide concentration range of the proteins, from micromolar to femtomolar level [70], and the presence of highly abundant proteins (e.g. albumin; immunoglobulins) that constitute more than 99% of the total protein amount. As a consequence, discovering and validating novel protein biomarkers for CVDs in plasma is very challenging [71], especially when the aim is the detection of gender-specific biomarkers.

Interest in gender differences in plasma dates back to the 1960s, when some papers described such aspects mainly in animal studies, such as in monkey and fish [72, 73]. More recently, studies performed on serum of cardiovascular patients have highlighted gender-related differences. Serum adipocyte fatty acid-binding protein (A-FABP) levels, for example, have a greater impact on atherosclerosis in women, being independently associated with carotid intima-media thickness, probably due to the higher fat percentage in women, to a difference in regional fat distribution, or to sex hormones regulation [74]. Furthermore, in the

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non-diabetic population, smoking associates differently with subclinical inflammation in the two sexes, with a decreased adiponectin level in women and with an increased hs-CRP level in men [75].

The first systematic proteomic study specifically addressing differences in serum protein composition between healthy male and female subjects, was conducted by Miike et al. in 2010 [76]. By removing highly abundant proteins and combining iTRAQ labeling, HPLC, nano-LC and MS, the authors succeeded in identifying and analysing 4000 proteins from the human serum. They found differences in the serum proteome of males and females: proteins more abundant in females participated in cascades commonly involved in female diseases, such as breast cancer and arthritis, whereas proteins more abundant in males were involved in hormonal response and were usually activated in conditions such as hypertrichosis and virilism [76].

To circumvent the limitations of immunodepletion-based strategies, which may lead to biases because of cross-reactions of the antibodies used or by proteins bound to carrier proteins such as albumin [77, 78], a subproteome enrichment by size-exclusion chromatography followed by iTRAQ 2D-LC-nESI-FTMS analysis of whole serum of obese adults was performed by Al-Daghri et al. [79]. Among the 2472 identified proteins, 248 proteins exhibited significant modulation between women and men. A key observation was the gender-specific differences in proteins associated with β-estradiol signaling and immune system, which were less abundant in males than in females, whereas the opposite occurred for proteins involved in lipid and testosterone metabolism, vitamin D signaling, and coagulation [79].

The utility of proteomics to identify disease markers is becoming increasingly evident in multifactorial diseases, such as CVDs, for which the value of using more than one marker has been highlighted in several studies [80, 81]. Zethelius et al. [80] suggested that a combination of biomarkers reflecting the myocardial cell damage (i.e. troponin I), left ventricular dysfunction (i.e. N-terminal pro-brain natriuretic peptide), renal failure (i.e. cystatin C), and inflammation (i.e. C-reactive protein) could improve the risk stratification with respect to a model essentially based on established risk factors. A more extensive study, including 47 selected markers of inflammation, lipoprotein metabolism, adipocyte metabolism, calcification and thrombosis measured by a multiplex immunoassay, was performed in 2561 men and women of African-

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American and non-Hispanic White ethnicity [81]. The authors reported an association between female sex and levels of inflammatory and calcification markers, insulin-resistance promoting adipokines, natriuretic peptides, and coagulation factor levels and activity, independently of potential confounding variables [81].

5.1.2 Urine

Another interesting biological fluid in proteomics is urine; similarly to plasma, it provides information not only from the urinary track, but also from other organs, potentially providing biomarkers for other systemic diseases. Moreover, urinary proteomics may be advantageous in terms of non-invasiveness of urine sampling, low dynamic range of analytes which facilitates the detection and analysis of biomarkers, lack of requirement for special sample preparation, and relative stability of the stored sample [82]. Of course, the interest in urinary proteome developed first in the field of urologic and kidney diseases, in particular in IgA nephropathy [83] and prostate cancer [84], but there is now an increasing interest in investigating urine as an orthogonal sample for studying systemic diseases [85]. Indeed, ongoing clinical trials involving urinary proteomics for protein biomarker discovery or validation (registered at clinicaltrials.gov) included studies in urologic and kidney diseases, as well as studies analysing urine along with orthogonal bodily fluids or tissue samples in diseases spanning neurology, cancer, and cardiology, among others [85].

The central question about individual variability or gender-related variations in the normal urine proteome was first addressed by Thongboonkerd et al. [86], who observed using two-dimensional electrophoresis (2-DE) that total protein was higher in male urine compared to female urine, but there were fewer protein spots. Recently, a study based on a 2D-LC-MS/MS and iTRAQ approach provided evidence of significant differences between the male and female urinary proteomes [87]. In particular, the females had higher abundance of some lipid and carbohydrate metabolism-related proteins. The analysis also revealed a larger inter-individual variation in the female urinary proteome than in males, maybe due to the higher variation in the levels of proteins associated with inflammatory response and cell movement and migration [87].

The analysis of the urinary proteome in females and males is extremely timely considering the diagnostic utility of the urinary proteomics in the cardiovascular field. In 2012, Kuznetsova et al. [88] found a panel of urinary proteins that were specific for essential hypertension with left ventricular dysfunction from a

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discovery set in asymptomatic hypertensive patients; this set also distinguished, in a validation test, hypertensive patients with HF from healthy controls. The same authors also found that, in the general population, the urinary proteome correlated with diastolic LV dysfunction [89], and that the urinary peptide-based classifier, but not systolic pressure, predicted the incidence of fatal and nonfatal cardiovascular and cardiac events over a follow-up period of 5 years in 791 randomly recruited Flemish subjects [90].

5.2 Tissue proteome

5.2.1 The aging myocardium

The first proteomic study with a particular emphasis on myocardial gender differences was performed in a primate model of aging heart and published by Yan et al. in 2004 [91]. By employing 2-DE coupled to mass spectrometry (2-DE/MS), the authors found that only in the left ventricular samples of male monkeys there was a decreased abundance of enzymes participating in glycolysis (e.g. pyruvate kinase, α-enolase), glucose oxidation (pyruvate dehydrogenase E1 β), the tricarboxylic acid cycle (oxoglutarate dehydrogenase), and the electron transport system (complexes III-V) accompanied by a reduced capacity of mitochondria for oxygen consumption. As these differences were also present in the human failing heart [92, 93], they could be involved in the pathogenesis of the disease, whereas the absence of these changes in females might explain their delayed cardiovascular risk.

5.2.2 Sex differences in pressure overloaded heart

Left ventricular hypertrophy (LVH), characterised by the growth in left ventricular mass caused by increased cardiomyocyte size, can be a physiological adaptation to strenuous physical exercise or a pathological condition, which is either genetic or secondary to left ventricular overload. While physiological LVH is usually benign and regressive, pathological LVH is a compensatory phenomenon, which eventually may become maladaptive and evolve towards progressive left ventricular dysfunction and HF. A large number of studies have recognized the influence of sex and/or gender on pathological cardiac remodeling and have shown differences in clinical outcomes and therapeutic responses, with males more prone than females to

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develop greater cardiac remodeling responses in hypertensive condition and aortic stenosis (reviewed in [94]). In the latter case, the cardiac performance is more preserved in female compared with male patients with a similar degree of aortic stenosis [95, 96]. Whether sex/gender-related differences result from intrinsic differences in molecular adaptation to pressure overload, or are related to age, degree of stenosis, left ventricle geometry or other factors extrinsic to the myocardium is not currently known. By employing the transverse aortic constriction model to simulate pressure overload in male and female wild-type (WT) and estrogen receptor β (ERβ) knockout mice, Kararigas et al. [97] found that in WT mice, hypertrophy was significantly more pronounced in males than females, an effect that was abolished in ERβ knockout mice, thus supporting the hypothesis of a cardioprotective effect of estrogen in pressure overload [98]. To provide mechanistic insights into the influence of sex and ERβ on the heart response to pressure overload, they used 2-DE/MS and found decreased levels of several metabolic and mitochondrial proteins, a finding compatible with the negative outcome in males. For example, males with pressure overload had a reduced level of aldehyde dehydrogenase, which has been shown to play a major role in cardioprotection and maintenance of contractile function in alcohol-induced left ventricular hypertrophy and ischemia/reperfusion injury [99, 100]. Furthermore, in male ERβ knockout mice with pressure overload there was a substantial decrease in the levels of several myosin heavy chain isoforms compared with the sham control group, suggesting an increased susceptibility of male ERβ knockout mice to impairments in the functional and structural adaptation to pressure overload. On the other hand, in female mice proteins that might confer cardioprotection, such as cytoskeletal and structural proteins, appeared to be elevated in response to pressure overload [98]. Vinculin, for example, is an important protein of the cytoskeleton, an actin-binding protein whose mutations can cause dilated cardiomyopathy in humans [101]. Thus, this proteomic analysis suggests that the response of the heart to pressure overload is highly modulated by sex and that ERβ is crucial for the tight regulation of mechanisms active in the development of left ventricular hypertrophy.

5.2.3 Cardioplegia

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Cardioprotection afforded by cardioplegia, a reproducible and safe method to induce and maintain electromechanical cardiac quiescence during surgeries, has been found to be significantly lower in the aged female compared with the aged male rabbit heart [102]. These findings are in accordance with human studies indicating that women have a significantly higher risk and worse outcomes after cardiac surgery with respect to men [103, 104]. Furthermore, in patients undergoing coronary artery bypass grafting (CABG), women have a significantly higher operative mortality and less favorable long-term survival than men [104]. Multivariate analysis also shows that women have higher mortality rates than men in low-risk and medium-risk groups. Only among very high-risk patients is gender not found to be an independent predictor of adverse outcomes [104]. Among possible mechanisms involved in these gender differences, mitochondrial function seems to be modulated by gender, as well as by age, suggesting a role in the gender-related responses to global ischemia and to the cardioprotection afforded by cardioplegia [105]. A proteomic report by Black et al. [106] showed that specific pathways associated with the mitochondrion modulated cardioprotection using cardioplegia in the mature rabbit male and female hearts. Specifically, glycolysis/gluconeogenesis and the pentose phosphate pathway were affected in the aged male hearts, whereas glyoxylate/dicarboxylate metabolism was significantly altered only in female hearts. The authors suggested that an alteration of these pathways might contribute to decreased myocardial functional recovery and myonecrosis following ischemia [106]. It is expected that improved understanding will pave the way to future cardioprotective approaches.

5.2.4 Atherosclerotic plaque

Notwithstanding the high heterogeneity of atherosclerotic lesions, which makes plaque analysis a challenging task, proteomic profiling of human plaque samples has been shown to be a feasible approach for the analysis of proteins within the atherosclerotic lesion [107, 108]. A variety of proteomics techniques have been used, from 2-DE with peptide mass fingerprinting, to more complex mass spectrometry techniques, utilising LC-MS/MS, or a combination of these techniques [107, 108]. Up to now, the only study that investigated the potential sexual dimorphism in plaque proteome was performed by Liang and colleagues, which used 2-DE combined with MALDI-TOF MS, as well as nLC-MS/MS for secondary

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confirmation, to analyse the proteomic profile of different regions of human carotid plaques [109]. Twenty six patients undergoing carotid endarterectomy were enrolled in the study, which had an equal gender ratio and very similar mean ages for men (72.6 ± 1.8 yrs) and women (71.4 ± 1.7 yrs). Different regions of human carotid plaques were studied, specifically fatty streak, plaque shoulder, plaque centre, and fibrous cap; these were compared to an internal control [109]. In this study, 2-DE/MS analysis identified 52 unique proteins, 41 of which were confirmed by nLC-MS/MS analysis, including proteins such as procollagen C-endopeptidase enhancer 1, biglycan, hepatoma-derived growth factor, calmodulin, SH3 domain-binding glutamic acid-rich like, and Protein S100-A11, which had not previously been mapped in human carotid plaques. By 2-DE/MS, the abundance of 18 proteins was found to be significantly altered in plaque regions compared to the internal control region. Of these proteins, 5 showed gender-specific alterations with 2-DE/MS, including ferritin light chain and transthyretin, which were also validated using nLC-MS/MS [109]. In men, a significantly higher content of ferritin light chain was detected in fibrous cap, in line with previous proteomic investigations showing an increased abundance of ferritin light chain in atherosclerotic plaque [110, 111]. In contrast, the abundance of ferritin light chain was found to be significantly decreased in female carotid plaque relative to the respective internal control site [109]. These findings deserve further investigation, since ferritin light chain is responsible for the storage of iron in cells and the accumulation of tissue iron has been implicated in the progression of atherosclerosis [112]. On the other hand, the content of transthyretin was found to be significantly higher in female carotid plaque [109]. Transthyretin is an evolutionarily conserved carrier protein associated with cardiac amyloidosis and a serine peptidase that is suspected to play multiple pathophysiologic roles, including the cleavage of substrates such as apolipoprotein A-I, that might affect the development of atherosclerosis [113, 114]. Its function within the atherosclerotic lesion and the significance of the gender difference in its abundance in human carotid atheroma, reported for the first time by Liang and colleagues [109], is unknown.

5.2.5 Adipose tissue

The first proteomic analysis addressing gender differences in visceral adipose tissue from type 2 DM patients was published in 2016 by Gomez-Serrano et al. [115]. Protein abundance changes reported in this

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study revealed distinctive male and female phenotypes in terms of the antioxidant response: levels of SOD1, SOD3 and several GST proteins were higher in men, and the peroxide-scavenging enzymes GPX1 and GPX3 were higher in women. Interestingly, the levels of fatty acid synthase were found to be increased in women, supporting the notion that visceral adipose tissue in women correlates with dysfunctional hypertrophic adipocytes characterised by a significant increase in cell size, in contrast to men who showed more numerous and smaller adipocytes (adipocyte hyperplasia). According to the authors, these novel findings suggest a worsening of the obese phenotype in women once type 2 DM emerges, due to an increased pro-inflammatory state and decreased visceral adipose tissue adipocyte hyperplasia compared to men, resulting overall in a more dysfunctional adipose tissue [115].

5.3 Circulating cells: platelets

That gender might influence platelet biology was anticipated over 30 years ago [116, 117]. A state-of-the-art paper by Patti et al. [118] highlights that, although less represented in clinical studies, the female gender may obtain different benefits from antiplatelet therapy with respect to men. Also, the thrombotic and bleeding risks, as well as outcomes after a cardiovascular event, appear to differ between genders. Among the multiple factors involved in these effects, hormonal mechanisms and differences in platelet biology might contribute to different gender characteristics. From a biochemical point of view, it is well known that many differences occur in platelets between females and males: the platelet count differs significantly, with higher values in women than in men [119]; in women, platelets have a higher number of surface receptors and bind a greater amount of fibrinogen (reviewed in [118]); their reactivity is also different, both with and without antiplatelet therapy [120]. The increased platelet responsiveness in females, at least in animal models, appears to be an intrinsic feature of the platelet itself, independent of the platelet size and the expression of surface adhesion molecules [121].

In recent years, it has become increasingly evident that proteomics can provide novel insights into basic research questions regarding the protein composition and the post-translational modifications (PTMs) occurring in platelets, which might be useful to understand the impact of the diseases and, eventually, of therapeutic interventions [122]. Indeed, platelet signaling is much more complicated and nonlinear than

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originally anticipated, involving a considerable level of cross talk among signaling pathways. However, as yet no proteomic study has addressed the issue of sex/gender-specificity of platelet proteomics in the cardiovascular system, with the exception of a single paper on human platelets derived from volunteers [123]. In this study, using protein microarrays, Eidelman et al. showed that gender differences appeared in the low abundance signaling proteome, whereas 2-DE revealed only high abundance proteins that did not differ between genders. Considering that there are still gaps in knowledge on gender-specific platelet biology and antiplatelet therapy, and that the percentage of women included in clinical trials evaluating the impact of antiplatelet drugs on cardiovascular outcomes (≈30%) has not changed in the last 20 years, it is evident that more women need to be included in order to produce strong evidence-based recommendations on the topic.

5.4 Gender dependence of nutritional effects on proteomics

In the nascent arena of nutriproteomics, proteomics aims to characterize the molecular and cellular changes occurring at the protein level following exposure to food nutrients. Indeed, proteomics in nutritional sciences can help indeed to understand the impact of nutrients on living systems, to identify potential biomarkers that can aid in lifestyle changes or dietary habits, and, finally, to assess food safety and functionality [124].

As discussed by Anand et al., short-term controlled-feeding studies with CVD risk factors as outcomes, long-term prospective cohort studies with CAD, stroke, and type 2 DM as outcomes, and a limited number of randomized controlled trials with CVD endpoints collectively show that multiple aspects of diet substantially influence CVD risk [125]. This review, as many others [126, 127] underlines that the traditional Mediterranean-type diet, characterised by a high intake of olive oil, fruit, nuts, vegetables, and cereals; a moderate intake of fish and poultry; a low intake of dairy products, red meat, processed meats, and sweets; and wine in moderation, provides a well-tested healthy dietary pattern to reduce CVD risk by about a third. A series of papers by Bedard [128-131] showed that men have greater cardiometabolic changes than premenopausal women in response to the Mediterranean diet. Up to now, some proteomics studies

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have been performed to investigate the effects of nutrients typical of the Mediterranean diet, i.e. olive oil and omega-3 fatty acids, on different specimens (lipoproteins, urine, peripheral blood mononuclear cells, platelets) in the context of CVDs [132-138], but no one has specifically addressed sex or gender differences.

Beyond the impact of food on the proteome, there is convincing evidence, at least from animal models, that caloric restriction benefits health by slowing the aging process and delaying the onset of age-associated chronic diseases, including CVD. Several studies have now demonstrated that cardiovascular aging can be affected by changes in food intake [139, 140], mainly due to reductions in inflammation and oxidative stress: in the vasculature, caloric restriction appears to protect against endothelial dysfunction and arterial stiffness and attenuates atherogenesis by improving several cardiometabolic risk factors; in the heart, it reduces cardiomyocyte apoptosis, protects against fibrosis, and preserves or improves left ventricular diastolic function [141]. While there is strong evidence supporting the inclusion of modest caloric restriction in lifestyle programs targeting cardiovascular health, the impact of caloric restriction on human health is not fully understood and deserves further investigation [142].

The aging process is further complicated by the sex differences in lifespan, which is a world-wide phenomenon with women outliving men by more than a decade in some countries, and not unique to humans because most sexually reproducing species show sex differences in patterns of ageing. A comprehensive explanation does not currently exist, even if interference of sex-steroids and altered activity of nutrient-sensing pathways may contribute [143]. Up to now, only one paper has addressed the combined effects of gender and caloric restriction at the proteomic level. Valle et al. found that females differ remarkably from males in the mechanisms that regulate substrate utilization and energy metabolism, in the antioxidant systems, and in the stress response [144]. Caloric restriction also affects overlapping sets of proteins and many of the gender differences are attenuated by caloric restriction suggesting that cellular pathways are similarly regulated in females and caloric restricted rats and could be related with a greater longevity [144].

6. Gender differences in oxidative stress and oxidative PTMs in CVDs

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6.1 Oxidative stress in CVDs

Partially reduced oxygen species (PROS; e.g. superoxide radical anion, hydrogen peroxide, hydroxyl radical) and oxynitro species (e.g. nitric oxide, peroxynitrite) at physiological levels play an important role as regulatory mediators in fundamental cell functions and contribute to the maintenance of cell homeostasis [145]. In contrast, a redox imbalance in favor of pro-oxidant processes leads to oxidative stress and oxidative damage, which have been implicated in the pathogenesis of a wide variety of diseases including cancer, neurodegenerative diseases, and vascular diseases [145]. In particular, oxidative stress is one of the most potent inducers of endothelial dysfunction and is involved in the initiation, progression and clinical manifestation of atherosclerosis [146-148]. Moreover, several conditions that represent risk factors for CVD, such as hypertension, diabetes mellitus, metabolic syndrome, obesity and cigarette smoking, are strongly linked to oxidative stress [149].

Evidence is emerging for gender differences in the occurrence and susceptibility of redox imbalance and oxidative stress, including in the cardiovascular system. For example, gender differences have been found in circulating leptin, which has proinflammatory properties, and leptin levels were found to correlate with increased total glutathione [150]. Markers of oxidative stress have mostly been reported to be lower in females than males during the first decades of life, but oxidative stress appears to be elevated in post-menopausal women when compared to pre-menopausal women, and is thought to play a major role in menopause symptoms such as hot flushes and osteoporosis, which argues for the involvement of female sex-hormones in maintaining low oxidative status [151]. In elderly people the redox balance seems to be inverted; for example, higher serum hydroperoxide levels have been observed in female CAD patients compared to males with CAD [151, 152]. As a result, it has been suggested that estimation of oxidative stress could be a useful biomarker for cardiovascular risk especially in elderly women [153]. However, “oxidative stress” is a composite of many different parameters and therefore comes in a variety of forms; there is no single universal measure. This emphasizes the importance of using a panel of redox biomarkers appropriate to the disease condition [154]. In atherosclerosis and other vascular dysfunctions, the presence

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of lipid oxidation products such as lipid hydroperoxides, small reactive aldehydes (malondialdehyde, hydroxynonenal) or oxidized LDL have commonly been analysed by a variety of methods [149, 155]. Oxidative stress can lead to oxidative modifications of proteins, with a variety of (mostly deleterious) effects on the functions of those proteins, and it is increasingly appreciated that a full understanding of the proteome and how it is altered by physiological conditions (such as gender) or disease requires analysis of all different protein forms, as discussed below.

6.2 PTMs and protein speciation in CVDs

The existence of PTMs and their effect on protein function has been recognized for many years: for example, phosphorylation is an archetypal PTM that regulates activity of many enzymes and interactions of proteins, but the number and types of PTMs existing is now known to be very extensive [156]. However, many proteomics studies simply seek to identify proteins in samples, and the issue of variability in protein structure is thus often ignored. This approach has significant limitations for understanding cellular processes, as explained by Jungblut et al. (2008), who coined the termed "protein speciation" to reflect the enormous variety in protein chemical structures over and above the amino acid sequence [157]. "Protein species" are defined as different protein forms resulting from covalent modifications of the protein with functional relevance [158]; they are thought to occur for most mammalian genes, and it has been estimated that, while the number of genes encoding human proteins is approximately 20,000, the number of human protein species is in the range of 1 billion. Species variation arises at every step from gene expression to protein degradation, and influences subcellular localization, degradation, subunit assembly, tertiary structure or enzyme activity.

Nowadays it is clear that information at the protein species level cannot be ignored to obtain biological relevant information on a protein. Indeed, the success rate of FDA approved diagnostic markers to date is very low compared to the number of published disease markers, and it has been suggested by Steffen et al. [159] that the biochemistry of the proteins and especially the occurrence of a multitude of protein species originating from a single gene is a major reason for this. This is particularly important in the setting of heart disease, which comprises a diverse range of acute (such as ischemia/reperfusion), chronic (such as heart

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failure, dilated cardiomyopathy) and genetic (such as hypertrophic cardiomyopathy) disease states, all of which have been associated with protein PTMs [160]. These notions on relationships of function to the exact chemical formula of the protein species have recently been discussed in a Special Issue of the Journal of Proteomics (2016), with recommendations on how to improve studies of a proteome, particularly in the disease state, in the future [161]. In CVD research, the protein species concept has been introduced by Schwab et al., who performed a 2-DE/ESI-LC-MS approach to assess the effect of a dietary supplement with the phytoestrogen genistein on the protein patterns involved in the maintenance of normal heart physiology at the protein species level [162, 163]. By this approach, the authors observed a substantial impact of sex, age, and genistein on the abundance of a multitude of protein species, especially mitochondrial enzymes involved in the fatty acid metabolism or playing a role in the tricarboxylic acid cycle or the respiratory chain [163].

Because it is not possible in this review to consider all of the possible PTMs, the next sections focus on oxidative modifications, as there is growing interest in proteomics and analysis of oxidative PTMs (oxPTMs) to proteins in CVDs. While many oxidative modifications to proteins can occur, including oxidations of cysteines, methionines, prolines, as well as hydroxylations, chlorinations and nitrations of tyrosines or tryptophans, only a subsection of these are thought to have regulatory effects [156]. Many other modifications may have no functional effect, or simply cause loss of activity. Another interesting category of oxPTMs are those caused by adduct formation by small reactive aldehydes, which includes the formation of AGEs (advanced glycation end products) and ALEs (advanced lipoxidation end products); some functional effects have been described for these modifications, such as altered binding to the receptor for AGEs or altered subcellular localization.

Analysis of all of these oxPTMs by mass spectrometry is extremely challenging [164], and this is especially true of glycation and lipoxidation [164-166]. Unlike enzymatically-induced modifications, such as phosphorylation, ubiquitinylation or farnesylation, which occur on specific residues, oxPTMs tend to occur randomly on a number of susceptible residues and proteins, making it extremely difficult to define all protein species. Thus improved enrichment processes and mass spectrometry-based methods for detection

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of oxPTMs including AGEs and ALEs are urgently required. Development of untargeted and semi-targeted bottom-up MS methods together with improved data mining algorithms are currently being developed with the H2020 innovative training network MASSTRPLAN (Project ID: 675132; http://cordis.europa.eu/project/rcn/198275_en.html), and will ultimately help to identify a larger fraction of protein species and their role in CVDs. However, at the current time most work on oxPTMs in CVDs have been carried out by more conventional proteomics approaches, as described in the following section.

6.3 Emerging role of oxPTMs in CVDs

In the context of CVDs, the interest towards PTMs and especially oxPTMs of proteins has grown considerably. The analysis of PTMs should provide useful information for the identification of mechanisms potentially involved in the genesis and/or progression of CVD. Ranging from immediate and reversible modifications, such as phosphorylation and some oxidative modifications, which enable rapid response to changes in the cellular environment, to long-term and irreversible modifications, such as AGE formation, analysis of the PTM status of proteins can provide clues to the molecular basis of the underlying pathology. Furthermore, emerging evidence supports a major role of PTMs in regulating multiple pathways of the intracellular quality control mechanisms evoked by the cell to minimize the level and toxicity of misfolded proteins and defective organelles in the cell. Indeed, poor quality control is associated with many forms of heart diseases [167, 168]. Liddy et al. [160] nicely described the most relevant PTMs that seem to be of emerging significance in cardiac disease, but within the Human Proteome Project further work is going on to identify and characterize as many PTMs as possible, including oxPTMs [169].

At the organelle level, the discovery and knowledge of PTMs occurring in the mitochondrial proteome have recently exploded with the advent of mass spectrometry and the most characterised PTMs and oxPTMs have been nicely reviewed by Stram et al. [170]. Many mitochondrial PTMs have a relevant role in signal transduction pathways, energy generation, apoptosis, autophagy, metabolism, and tissue response to ischemic injury, but the functional significance of the various mitochondrial PTMs in regard to their impact on the pathophysiology of disease remains an intense area of investigation [170]. Mitochondrial dysfunction almost certainly has a role in CVDs, such as stroke, HF, and cardiac ischaemia/reperfusion

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injury [171, 172]. Based on these assumptions, it is likely that prevention or reversal of mitochondrial damages might represent a potential target for the treatment of CVDs. At the time of writing, the only study that investigated PTMs in the mitochondrial proteome linked to gender differences was performed in rat hearts by Lagranha et al. [173], likely due to the lack of standardized methods to analyse mitochondrial proteome [174]. The authors found an increased phosphorylation of aldehyde dehydrogenase 2 and of the E2 subunit of alpha-ketoglutarate dehydrogenase in females, an event that may be responsible of the lower production of oxidants and of the cardioprotection of the female heart in the ischemia-reperfusion model.

7. Conclusions

The differences between females and males begin even before implantation of the zygote in the uterus and continue throughout prenatal development phases, in childhood and adulthood. These differences include diverse susceptibility to some diseases, such as certain types of cancer and autoimmunity, in which females have an overall higher susceptibility [175]. The existence of sexual diversities in the onset, manifestation, and outcome has now been recognised also in CVDs [6-8]. The endogenous causes of the sex differences observed in many diseases are largely unknown, and the situation in CVD research is not much different. Beyond environmental and social differences between men and women (e.g., occupational hazards, lifestyle, social stresses, access to healthcare) that can contribute to gender differences in CVDs, sex hormones and sex chromosomes have been found to account for some sex differences in CVDs [8, 10-12]. However, several gaps in our understanding of sex- and gender-related diversities in cardiovascular health still exist. The search for sex/gender-related mechanisms is further complicated by the still-increasing sex bias in preclinical research [14], despite the fact that in 2014 the NIH announced that sex should be considered as a biological variable in applications for preclinical research funding [15].

By generating large sets of molecular data, ‘omics technologies, including genomics/transcriptomics, proteomics, metabolomics, lipidomics and others, can provide deep biological insight into human health and disease. Applications of these technologies to investigations aimed at elucidating the causes underlying sex- and gender-related diversities in pathophysiology is a challenging task. While great technological

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progress has been made and some excellent bioinformatic methods are currently available for computational analysis, further improvements in the acquisition, storage, handling and integration of large volumes of data are needed [176]. In particular, a crucial aspect of 'omics studies on complex phenotypes such as CVDs is the collection of high-quality biological samples providing the basis for the creation of large data sets that can accurately incorporate the many sources of variability (including key variables as race/ethnicity, age, and sex/gender) into rigorous statistical models [176]. Notwithstanding these challenges, it is hoped that, by exploiting multi-omics approaches to integrate information about gene expression and protein species composition of an organism with metabolic fingerprints and lipid profiles (Figure 2), we will gain a more comprehensive understanding on how sex and gender impact cardiovascular health. This is an exciting field where 'omics approaches could make a significant contribution to precision medicine [177].

Finally, the current knowledge of the relationship between the function and the exact structural formula of protein species to health and disease suggests that the focus on disease-associated protein species in the future will bring to more specific disease markers. Last, the exact chemical composition including not one but every posttranslational modification and complete sequence coverage at the protein species level should be achievable with further progress in sample preparation techniques, especially concerning separation techniques at the protein level, mass spectrometry and algorithms for mass spectrometric data processing.

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Funding: This work was supported by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement number 675132.

Financial and competing interests disclosure

The authors report no conflict of interest.

In Press, Accepted Manuscript. https://doi.org/10.1016/j.jprot.2017.11.012

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Figure legends

Figure 1. Women-specific CVD risk factors. Women-specific conditions to consider in risk evaluation, diagnosis and treatment of CVDs include hypertensive disorders of pregnancy, gestational diabetes mellitus, preterm delivery, menopause, systemic autoimmune disease, breast cancer treatments, and depression.

Figure 2. 'Omics applications in cardiovascular research: unveiling sex/gender-differences in CVDs. The figure highlights the role of proteomics in biomedical researches that expand from the screening of early diagnostic and prognostic biomarkers to the investigation of the molecular pathways underlying CDVs. The deciphering of proteomes via protein speciation, and its integration with genomics/transcriptomics, metabolomics, and lipidomics, may reveal novel mechanisms responsible for sex/gender-differences in CVDs, thus providing new opportunities oriented towards precision medicine.

In Press, Accepted Manuscript. https://doi.org/10.1016/j.jprot.2017.11.012

Executive summary

Clinically important sex- and gender-related differences exist in risk factors, occurrence, management and outcomes of cardiovascular diseases (CVDs).

Traditional CVD risk factors affecting both men and women

- Hypertension
  - Slightly higher prevalence in men than in women
  - Uncertain whether the association between increments in systolic blood pressure and CVDs differs between men and women
- Dyslipidemia
  - Similar prevalence of elevated total cholesterol (TC) in men and in women
  - Similar TC-related risk of CHD in men and in women
- Diabetes mellitus (DM)
  - Similar prevalence in men and in women
  - Higher excess risk of coronary heart disease (CHD), stroke, heart failure (HF), and peripheral arterial disease (PAD) in diabetic women compared with diabetic men
- Excess body weight
  - Similar prevalence in men and in women
  - Similar association between body mass index (BMI) and CHD in men and in women
  - Hormone-related sex dimorphism in patterns of body fat storage and fat metabolism potentially affecting the relationship between excess body weight and CVD risk
- Cigarette smoking
  - Mortality from CVDs higher in women than in men who smoke
  - Higher risk of developing CHD in women than men with the same exposure to tobacco smoke
  - Similar beneficial effects of smoking cessation on CVD risk in women and in men

Women-specific CVD Risk Factors

- Pregnancy complications
  - Hypertensive disorders of pregnancy and gestational DM are important women-specific factors to consider in CVD risk assessment
- Age at menopause
  - Women who undergo menopause before age 50 or primary ovarian insufficiency have an increased risk of CVDs
  - Controversy remains regarding the cardiovascular effects of menopausal hormone therapy; the current consensus is that it should never be prescribed for the aim of preventing CVDs
- Emerging, non-traditional CVD risk factors in women
  - Preterm delivery, systemic autoimmune diseases, breast cancer treatments, and depression are new emerging factors that can affect CVD risk in women

Sex/gender-differences in CVD manifestations and underlying pathophysiology

- Coronary artery disease (CAD)
  - Prevalence higher in men than women at younger ages, but the incidence rises in women after menopause
  - Prevalence of obstructive CAD phenotype in men vs. non-obstructive CAD in women
  - Plaque rupture more frequent in men than in women; plaque erosion more frequent in premenopausal women than in men
  - Poorer prognosis in women compared to men
- CVDs more prevalent in women
  - Expanded spectrum of coronary disease in women, comprising coronary microvascular dysfunction (CMD), spontaneous coronary dissection (SCAD), and Takotsubo cardiomyopathy

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- HF (particularly with preserved ejection fraction) affects typically more women than men

- Ischemic stroke
  - Increased lifetime incidence in women compared with men
  - More severe strokes and greater disability in elderly women compared with age-matched men
- PAD
  - Prevalence higher in men than women at younger ages, but the incidence rises in women after menopause
  - More severe PAD in women compared to men
- Abdominal aortic aneurysm (AAA)
  - Prevalence higher in men than women
  - Worse outcomes in women

Role of oxidative stress in CVDs

- Several conditions that represent risk factors for CVDs are strongly linked to oxidative stress
- Oxidative stress is involved in the initiation, progression and clinical manifestation of atherosclerosis
- Evidence is emerging for gender differences in the susceptibility to oxidative stress
- Oxidative stress can lead to oxidative modifications of proteins (oxPTMs), with a variety of (mostly deleterious) effects on their functions
- oxPTMs, together with other post-translational modifications, are actually an intense area of investigations by proteomics

Gender proteomics in CVDs

- Applications of 'omics technologies to investigations aimed at elucidating the causes underlying sex- and gender-related diversities in pathophysiology is a challenging task
- A full understanding of the proteome and how it is altered by physiological conditions (such as gender) or disease requires analysis of all different protein forms
- Proteomic studies in this field are still very rare, but they are expected to increase over the next years

Several gaps in our knowledge of sex/gender-related diversities in CVDs still exist. The successful integration of 'omics technologies (including genomics/transcriptomics, proteomics, metabolomics, lipidomics, and others) could make a significant contribution to precision medicine developed on top of sex/gender-based assessments.

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SEX VS. GENDER

Sex and gender are different constructs. According to the WHO, sex *"refers to the set of biological characteristics that define humans as female or male"*; it is primarily associated with physical and physiological features including chromosomes, gene expression, hormone levels, and reproductive/sexual anatomy. Gender encompasses biology but is also influenced by experience and environment: it *"refers to the socially constructed roles, behaviors, activities, and attributes that a given society considers appropriate for men and women"*; it influences the distribution of power and resources, including access to healthcare. Sex and gender influence each other through complex interactions. Both sex and gender are critical variables in preclinical and clinical research.

http://www.who.int/reproductivehealth/topics/sexual_health/sh_definitions/en/

http://www.who.int/gender-equity-rights/understanding/gender-definition/en/

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Nonstandard Abbreviations and Acronyms

2-DE, two-dimensional electrophoresis

2D-LC-MS/MS, two-dimensional liquid chromatography/tandem mass spectrometry

2-DE/MS, two-dimensional electrophoresis coupled to mass spectrometry

8-plex iTRAQ

AAA, abdominal aortic aneurysm

BMI, body mass index

CAD, coronary artery disease

CHD, coronary heart disease

CMD, coronary microvascular dysfunction

CVD, cardiovascular disease

DM, diabetes mellitus

ERFC, Emerging Risk Factors Collaboration

ERβ, estrogen receptor β

GPX, glutathione peroxidase

GST, glutathione S-transferase

HDL-C, high-density lipoprotein cholesterol

HF, heart failure

HR, hazard ratio

IHD, ischemic heart disease

iTRAQ, isobaric tags for relative and absolute quantitation

LVH, left ventricular hypertrophy

MALDI-TOF MS, matrix-assisted laser-desorption ionization- time of flight mass spectrometry

NIH, National Institutes of Health

nLC-MS/MS, nano liquid chromatography/tandem mass spectrometry

oxPTM, oxidative post-translational modification

PAD, peripheral arterial disease

PTM, post-translational modification

SBP, systolic blood pressure

SCAD, spontaneous coronary dissection

SOD, superoxide dismutase

TC, total cholesterol

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Fig. 1

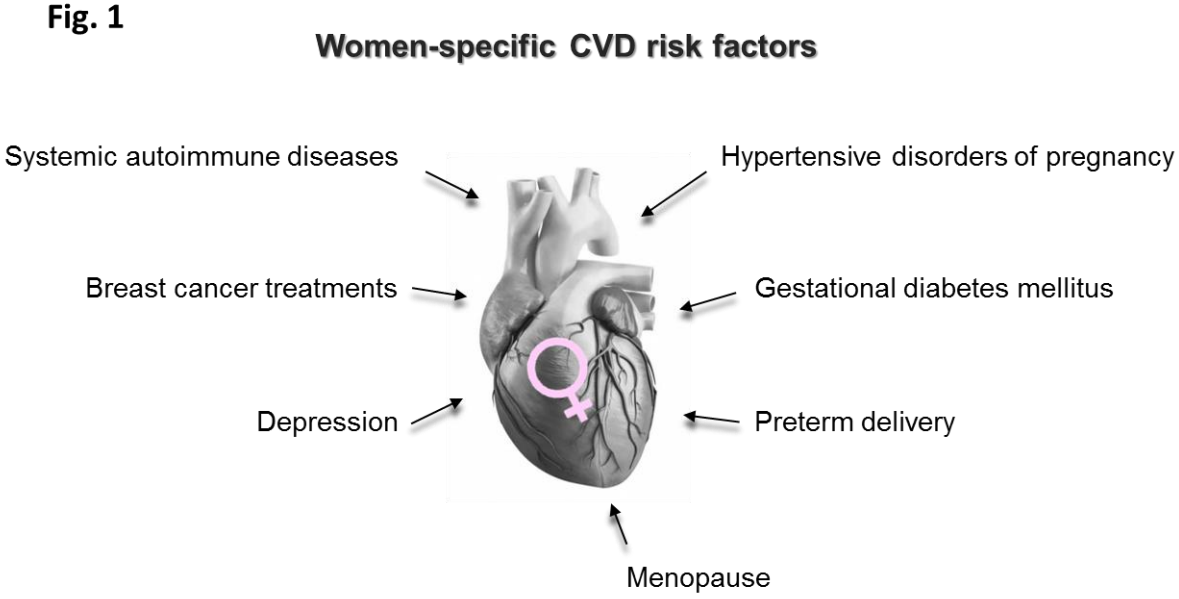

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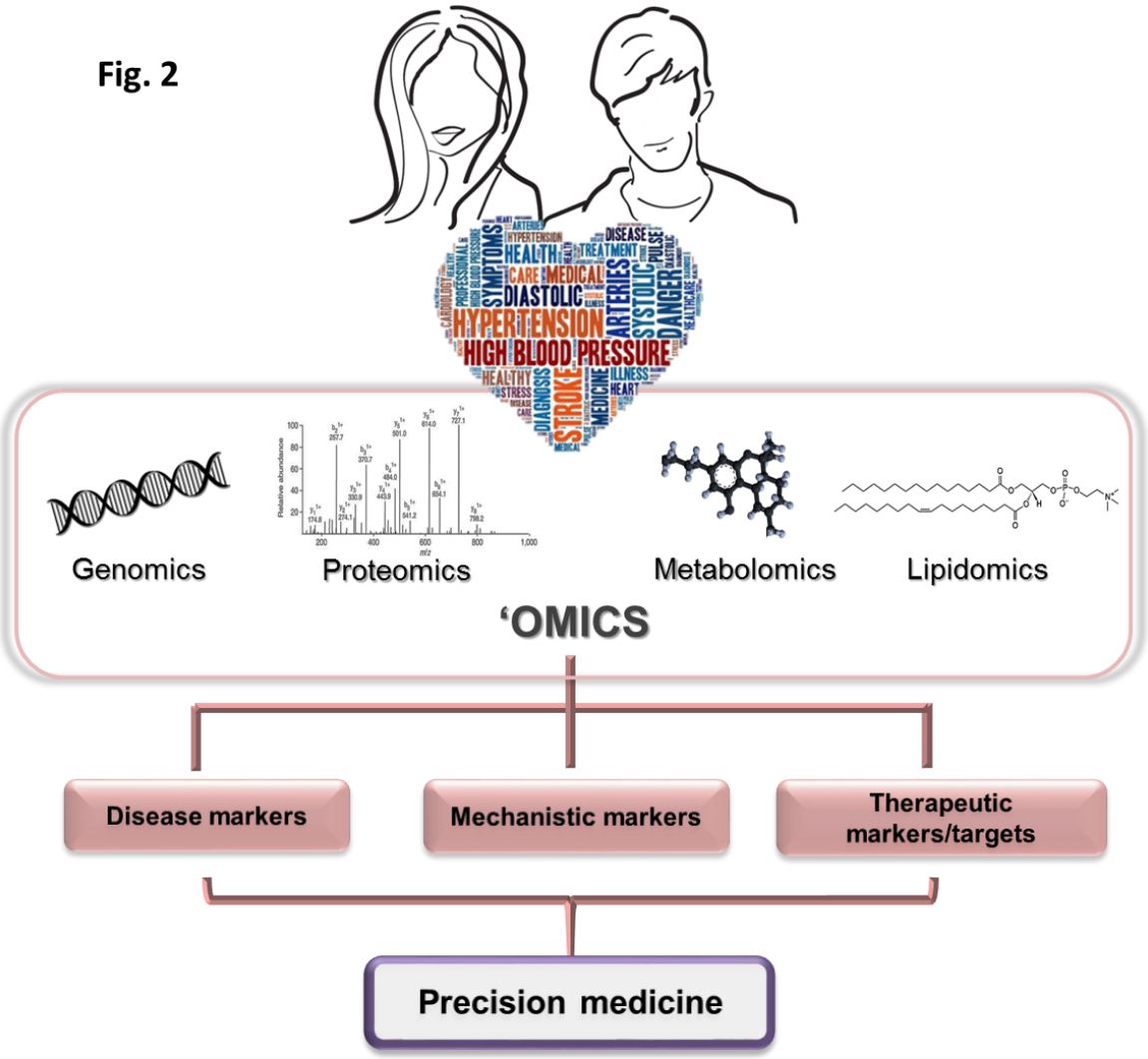
Fig. 2



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