Search for biomarkers of abdominal aortic aneurysm

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La verdadera ciencia enseña,
por encima de todo,
a dudar y a ser ignorante.

Miguel de Unamuno, escritor y filosofo español.

(La veritable science enseigne,
par-dessus tout,
à douter et à etre ignorant.)

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List of publications:

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Cieniewski-Bernard C, **Acosta A**, Dubois E, Lamblin N, Beseme O, Chwastyniak M, Amouyel P, Bauters C, Pinet F. Proteomic analysis in cardiovascular diseases. Clin Exp Pharmacol Physiol. 2008, 35:362-366.

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LIST OF ABREVIATIONS

I. List of abreviations:

2D-PAGE: two dimensional polyacrylamide gel electrophoresis

AAA : abdominal aortic aneurysm

AOD: athero occlusive disease

CID: collision induced dissociation

Cy: cyanine

DIGE: difference gel electrophoresis

EC-SOD: extracellular superoxide dismutase

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

ESI: electrospray ionization

ETD: electro transfer dissociation

EVAR: endovascular aortic repair

HUPO: human proteome organization

ICAM: intracellular adhesion molecule

IFN: interferon

IgA-CP: immunoglobulins agains Chlamidophila pneumonia

IL-4: interleukin 4

ILT: intraluminal thrombus

INF-γ: interferon gamma

iTRAQ: isobaric tag for relative and absolute quantitation

LC: liquid chromatography

LDL-cholesterol: low density lipoprotein

m/z: mass to charge ratio

MALDI: matrix-assisted laser desorption/ionization

MCP-1: monocyte chemotactic protein 1

MMP: matrix metalloproteinase

Mr: molecular weight

MS: mass spectrometry

NGAL: neutrophil gelatinase associated lipocalin

OAR: open aortic repair

OxLDL: oxidized low density lipoprotein

PAcIFIC: precursor acquisition independent from ion count

pI: isoelectric point

PICP: carboxyterminal propeptide of type I procollagen

PIIINP: aminoterminal propeptide of type III procollagen

PMF: peptide mass fingerprinting

PTM: post-translational modification

ROS: reactive oxygen species

SIL: Stable isotope labeling

SMC: smooth muscle cell

TGF: transforming growth factor

Th1: T-helper type 1

Th2: T-helper type 2

TIMP: tissue inhibitor of MMP

TMT: tandem mass tags

TNF: tumor necrosis factor

TOF: time of flight

FOREWORD

II. FOREWORD

Abdominal aortic aneurysm (AAA) is characterized by an increase of diameter (>1.5 times to reference diameter) and loss of parallelism of the vessel wall. This disease is more often asymptomatic and its rupture is responsible for 1-4% of mortality in males older than 65 years. Surgery treatment is possible in the case of detection by echography but no specific biological markers are available.

Biomarker discovery for this pathology needs to be performed in human studies because the available animal models do not entirely reflect the biomolecular mechanisms involved in the pathophysiology of AAA. Ideally, samples to be analyzed should be samples that reflect disease phenotype, for instance, the main cellular types involved in the pathology (macrophages and smooth muscle cells), as well as samples that reflect the application of biomarker detection, such as plasma or serum samples.

Differential proteomic analysis seems to be the technique of choice for biomarker discovery since samples are analyzed without any *a priori* hypothesis, allowing screening of a large range of proteins that can give rise to the identification of new biomarker candidates. Furthermore, differential proteomic analysis between diseased and control samples may allow a better comprehension of mechanisms involved in AAA through the identification of proteins implicated in the pathology.

INTRODUCTION

III. INTRODUCTION

1. Biomolecular and clinical aspects of abdominal aortic aneurysms

1.1. Definition

As a general definition, an aneurysm is a permanent focal dilation of a vessel to 1.5 times its normal diameter (Figure 1).

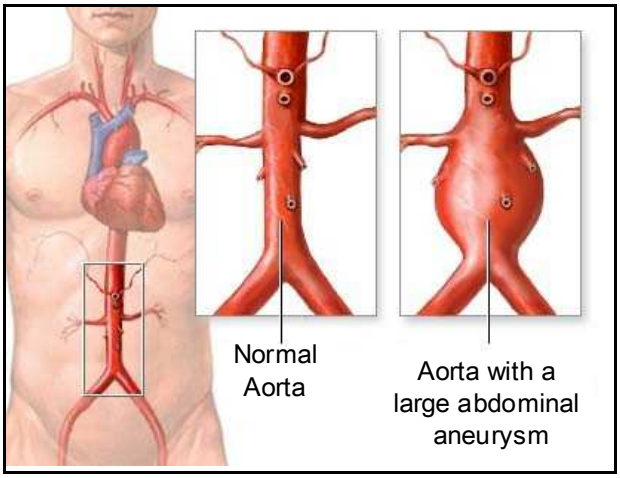

Figure 1. Localization of normal abdominal and aneurismal aorta.

Thus, abdominal aortic aneurysm (AAA) is the vascular pathology characterized by an increase of vessel diameter to at least 1.5 times the diameter of reference and a loss of parallelism of the aortic wall at the infrarenal region. This morphological definition, used currently for diagnosis, is now challenged by a more pathophysiological one: the loss of function of blood retaining by the arterial wall related to biological events taking place within it [Michel *et al.* 2008]. Irrespective of the definition, the underlying complication in abdominal aortic aneurysm is the weakening of the aortic wall, resulting in progressive dilation and, left untreated, eventual aortic rupture, which is often a fatal event. Depending on the gender, normal human aortic diameters in that region are between 1.5 and 1.7 cm. When aortic diameter exceeds 3.0 cm the aorta is considered aneurismal [Lederle *et al.* 1997b]. Abdominal aortic aneurysms larger than 5.5 cm in men and 4.5 cm in women are recommended for elective repair due to the high risk of rupture [Lederle *et al.* 2002; Brewster *et al.* 2003].

1.2. Epidemiology and risk factors

1.2.1. Epidemiology

Regarding epidemiology, AAA primarily affects elderly males with a prevalence of 5%. AAA rupture is responsible for 1-4% of the total mortality in males older than 65 years. In case of rupture the mortality is up to 70-95%. Moreover, mortality occurs in 65-75% of patients

before they arrive at hospital and in up to 90% before they reach the operating room [Brown *et al.* 1999]. Thus, AAA comes up as one of the leading causes of death in industrialized countries with aging populations. For instance, in 2000 it was the 13th most common cause of death in the Western world [Thompson *et al.* 2000]. AAA causes around 15,000 deaths in the USA and 8,000 in the United Kingdom per year [Kuivaniemi *et al.* 2008].

Several large-scale epidemiologic studies have provided interesting insights into risk factors associated with the development of AAA. Advanced age, male gender, cigarette smoking, hypertension, genetic susceptibility and the presence of another atherosclerotic localization have been described as known risk factors to develop AAA [Alcorn *et al.* 1996], although the mechanisms of action of AAA and the relationship with these factors are not completely elucidated [Wassef *et al.* 2007].

1.2.2. Risk factors

AAA risk increases significantly with age. For example, in men aged 65-69 years, AAA incidence is 4.8% while in those aged 80-83 years incidence reaches 10.8% [Jamrozik *et al.* 2000]. Furthermore, it has been reported that subjects who are more than 75 years old have nearly 8 times the risk of AAA than subjects between 65 and 69 years of age [Forsdahl *et al.* 2009].

Together with advanced age, being male has been considered as one of the non-modifiable risk factors for AAA [Golledge *et al.* 2006]. However, a recent discussion about the possibility of increased prevalence of AAA in women came out due to an analysis of different definitions of aneurismal aorta. It has been demonstrated that depending on the definition of an AAA used, the prevalence in women could be much higher than currently thought. In the study performed by Wanhainen *et al.* [Wanhainen *et al.* 2001], depending on the diagnosis criteria, the AAA prevalence in women was between 0.8% and 9.4%. It could be reasonable to assume that the definition of 3.0 cm for the average diameter is inappropriate and leads to an underestimation of the prevalence of AAA. In addition, the risk of rupture in female patients with AAA between 5.0 to 5.9 cm is up to four-time higher than in male patients with AAA of the same diameter size, suggesting that a lower threshold for diagnosis and surgery should be considered in women [Brown *et al.* 2003]. Moreover, some population based studies which focused on female populations reported the association of advanced age and cigarette smoking with AAA development previously reported in men [Derubertis *et al.* 2007; Lederle *et al.* 2008]. Differences between men and women regarding epidemiology, etiology, risk of rupture and treatment of AAA were therefore recently reviewed [Grootenboer *et al.* 2009].

Cigarette smoking is the most important environmental risk factor, bearing the strongest association with the presence and continued expansion of AAA [Lederle *et al.* 1997a; Singh *et al.* 2001; Brady *et al.* 2004]. Current smokers are 7.6 times more likely to have AAA than non-smokers and ex-smokers are 3 fold more susceptible to AAA than non-smokers [Wilmink *et al.* 1999]. The latest update in the study of the population of Tromso in Norway (The Tromso Study), with detailed data about cigarette smoking, showed that subjects who currently were smoking 20 cigarettes or more per day had a more than 13 times increased risk of an incidence of AAA than subjects who had never been daily smokers [Forsdahl *et al.* 2009]. However, the relationship between smoking and the mechanisms of AAA formation and progression are still unclear. It seems that smoking could promote the expression of the proteolytic system involved in aortic wall degradation while simultaneously attenuating the activity of their inhibitors [Kakafika *et al.* 2007]. In any case, that does not explain the strong association of smoking with most of the aortic diseases. Interestingly, an increased risk for AAA with increasing alcohol consumption was recently reported [Wong *et al.* 2007].

Regarding hypertension, the problem is that its definition is often based on whether the patient is receiving treatment for this condition [Alcorn *et al.* 1996] [Pleumeekers *et al.* 1995; Singh *et al.* 2001; Jamrozik *et al.* 2000]. Thus, evaluation of the correlation between blood pressure and AAA is complicated. Many epidemiological studies found an absence of relationship between hypertension and AAA [Alcorn *et al.* 1996; Blanchard *et al.* 2000; Lee *et al.* 1997; Bengtsson *et al.* 1991; Lindholm *et al.* 1985; Wanhainen *et al.* 2005], whereas others described hypertension to be associated with AAA [Jamrozik *et al.* 2000; Singh *et al.* 2001; Vardulaki *et al.* 2000; Tornwall *et al.* 2001; Forsdahl *et al.* 2009; Brown *et al.* 1999]. Two studies that used experimental models of hypertensive rats to perform elastase-induced AAA showed that the size of AAA was significantly increased in hypertensive rats compared to normotensive rats [Gadowski *et al.* 1993; Shiraya *et al.* 2006]. However, in a more recent study on hypercholesterolemic mice it was demonstrated that AAA formation resulting from infusion of ANG II occurs independent of blood pressure-elevating effects of this peptide. In other words, it was demonstrated that blood pressure per se was not a major determinant of angiotensin II-induced AAAs [Cassis *et al.* 2009]. Both epidemiological human studies and animal models have reported controversial results concerning the relationship between hypertension and development of AAA even if generally it is considered to be a major risk factor.

1.2.3. Genetic factors

Genetic factors may also be involved in the development of AAA. Evidence ranges from differences observed in AAA prevalence between populations from different ethnic groups to the appearance of familial cases of AAA. Few population-based prevalence studies assessing AAA in non-Caucasians populations have been done until now. LaMorte et al. [LaMorte *et al.* 1995] described for the first time that atherosclerosis was more common in African than Caucasian Americans, but aortic aneurysm was more common in the Caucasian population. Later, African ethnicity was reported as negatively associated with the prevalence of AAA [Lederle *et al.* 1997a; Golledge *et al.* 2006] and prevalence of AAA among African-American men was described to be 39% less than Caucasian American men [Prisant *et al.* 2004]. Interestingly, within the Asiatic population and in the western pacific region the prevalence of AAA looks highly heterogeneous. Epidemiological study of different Asiatic ethnic groups settled in Borneo Island, Malaysia, showed that AAA in Asian population is not uncommon and the incidence is comparable to the Western world [Yii 2003]. Moreover, in one Australian study, subjects with Mediterranean origin were found to have a 40% lower risk of AAA than subjects with Australian origin [Jamrozik *et al.* 2000]. In contrast, two studies that took place in UK, in Bradford City [Spark *et al.* 2001] and Birmingham [Hobbs *et al.* 2006], assessed respectively AAA prevalence and incidence of AAA repair in Asian communities compared to the Caucasian population. In the first one, where Asians represent 30% of the total population of Bradford City, no cases of AAA were identified in the at risk Asian population, suggesting that AAA is rare among Asians. The second one, in which Asians were mostly Kashimiri-born Pakistani Muslims, reported that Asians were 10 times less likely to undergo AAA repair than Caucasian men and that the reduced incidence of surgery for AAA in UK Asians may be due to a low prevalence of disease. Regarding familial abdominal aortic aneurysm, positive associations with family history of AAA have been described in epidemiological studies [Norrgard *et al.* 1984; Johansen *et al.* 1986] [Jaakkola *et al.* 1996; Rossaak *et al.* 2001]. Clifton, who described familial AAA that affected three brothers of one family, was the first to hypothesize that AAA could be an inheritable disease [Clifton 1977]. It has been estimated that around 15% of AAA patients have a family history of AAA disease [Thompson *et al.* 2008]. Furthermore, the risk is around 11 times higher in subjects with positive family history of AAA in a first degree relative [Johansen *et al.* 1986]. This may be explained by similar environmental and socioeconomic factors like smoking or exercise and dietary habits. However, in multivariate analysis accounting for these factors, family history still appears to be a significant and independent risk factor for AAA [Blanchard *et al.* 2000].

1.2.4. Diabetes

Regarding diabetes, there is a general agreement when talking about its relationship with AAA. A negative association between diabetes and the prevalence of AAA has been reported from epidemiological studies [Lederle *et al.* 1997a; Wanhainen *et al.* 2005; Golledge *et al.* 2006], and these results have been shown in both male and female [Lederle *et al.* 2008]populations. Most often, the interaction of diabetes and AAA results in reduced expansion rate of aneurismal diameter. Recently, a possible mechanism to explain this relationship was proposed by Golledge et al. [Golledge *et al.* 2008a]. It is known that extracellular matrix (ECM) proteins play an important role in vessel remodeling; and it is also known that diabetes produces advanced glycation of ECM proteins such as collagen, resulting in changes in the three-dimensional structure of the ECM. Since cellular responses are influenced by the structural form in which the proteins are presented, Golledge et al. [Golledge *et al.* 2008a] hypothesized that glycation could cause structural changes to the aortic media that inhibited monocyte activation into macrophages and further secretion of matrix metalloproteinases (MMPs), delaying degradation of ECM and expansion of aneurismal aorta.

1.3. Molecular mechanisms

In most cases, AAA is considered to be one complication of atherosclerotic lesions due to a progressive degeneration of the aortic wall. However, the origin of AAA formation is not yet clear. Despite this lack of information, it is known that there are three main factors implicated in AAA formation and progression: 1) proteolytic degradation of the aortic wall connective tissue by matrix metalloproteinases (MMPs), 2) aortic wall inflammation, and 3) oxidative stress [Diehm *et al.* 2007].

1.3.1. Anatomical considerations of the aorta

Some anatomical considerations have to be explained in order to better understand and link together these mechanisms. From the three layers of the aortic wall structure, the tunica media is considered to be the most important component since it is the thickest layer. It consists mainly of smooth muscle cells (SMCs) with elastic layers in a collagen network. Collagen contributes tensile strength and prevents overdissection whereas elastin gives arterial wall distensibility on pulse propagation [Diehm *et al.* 2007]. In the normal situation, there is a gradual but substantial reduction in the number of media elastin layers along the aorta that goes from 60-80 layers in the proximal thoracic region to 28-32 layers in the infrarenal aorta [Wolinsky *et al.* 1969; Halloran *et al.* 1995]. Moreover, there is a reduction in both collagen and elastin content between the suprarenal and the infrarenal aorta. It was also

noted that the infrarenal aortic segment is the only location within the aorta where proportion of elastin decreases relative to collagen [Halloran *et al.* 1995]. Thus, all these anatomical observations help explain why aneurysms seem to have predilection for the abdominal region of the aorta.

1.3.2. Extracellular matrix degradation by matrix metaloproteinases

The main structural elements in the aortic wall are elastin and interstitial collagens. From the 23 members of the MMP family that have been found in humans, 19 are able to digest some of the different forms of collagen or elastin [Raffetto *et al.* 2008]. MMPs are specifically distributed in different tissues or cell types and have been described to be involved in several pathologies. There are research studies that have shown an increase of expression of MMP with elastolytic and collagenolytic properties in both human [Annabi *et al.* 2002; Abdul-Hussien *et al.* 2007] and experimental [Godin *et al.* 2000; Rush *et al.* 2009] AAA. Specifically, upregulation of MMP-2, MMP-9 [Sakalihasan *et al.* 1996; Wassef *et al.* 2001; Thompson *et al.* 2002; Annabi *et al.* 2002; Longo *et al.* 2002; Nishimura *et al.* 2003] and MMP-12 [Wassef *et al.* 2001; Thompson *et al.* 2002; Annabi *et al.* 2002] in the media seems to be implicated in AAA development. While MMP-2 and MMP-9 are expressed in many types of tissues and cells, MMP-12 is specifically secreted by inflammatory macrophages [Shapiro *et al.* 1993], which are associated with several destructive diseases. Indeed, the important implication of MMP-12 and macrophage recruitment in AAA development has been shown in a recent study in which elastase-induced AAA rats were treated with atorvastatin [Shiraya *et al.* 2009]. This drug, generally used for decreasing the level of LDL-cholesterol, seemed to inhibit the expression of ICAM and MCP-1, and, as a consequence, the recruitment of inflammatory macrophages into the aortic wall. Specifically, MMP-12 levels were significantly lower and synthesis of elastin and collagen significantly higher after treatment with atorvastatin. Other MMPs like MMP-3, MMP-8 and MMP-13 are specifically described to cause adventitia collagen degradation [Rizas *et al.* 2009]. Also, overexpression of MMPs causes an altered balance between them and their inhibitors called tissue inhibitors of MMP (TIMPs). This imbalance was shown by Knox *et al.* [Knox *et al.* 1997] in human aortic biopsies from both atherosclerotic and aneurismal tissues. These enzymes are secreted within the aortic wall by both macrophages and SMC and may also play a key role in AAA progression. For instance, overexpression of TIMP-3 promotes SMC death by apoptosis [Baker *et al.* 1998], generating so a decrease in ECM regeneration since SMC are the only cells that able to synthesize collagen and elastin. Another research study demonstrated that TIMP-1 play an important role interacting with MMP-2 and/or MMP-9 during development of aneurysm and TIMP-2 may be involved in the early stages of AAA

[Nishimura *et al.* 2003]. Interestingly, another study in TIMP-2 deficient mice showed that targeted deletion of this inhibitor results in attenuation of aneurysm development [Xiong *et al.* 2006]. While MMP-2 needs TIMP-2 as a cofactor to become active, at higher concentrations TIMP-2 is inhibitory to MMP-2. So, despite its name, the role of TIMP-2 in promoting aneurismal enlargement suggests that molecular mechanisms involved in ECM degradation are extremely complex and more studies in both human and animal models are needed to introduce new pieces into this jigsaw puzzle.

1.3.3. Inflammatory responses

A second important pathologic feature of human AAA is the infiltration of inflammatory cells, including monocytes, lymphocytes, and plasma cells in the media and adventitia layers of the vessel wall. Interestingly, some studies supported the hypothesis that T-helper type 1 (Th1) immune responses prevail in aneurismal lesions [Galle *et al.* 2005; Middleton *et al.* 2007] while another group demonstrated that there is a predominant T-helper type 2 (Th2) response in aneurismal tissue as opposite of a predominant Th1 response in atherosclerotic lesions [Schonbeck *et al.* 2002]. Later, the same group used an immunologically driven model of aneurysm formation in mice to demonstrate that specific blockade of interferon gamma (INF-γ) signaling (meaning depletion of Th1 response) and/or augmented interleukin-4 (IL-4) (i.e. due to predominance of Th2 response) can directly modulate elastolytic enzymes and yield degradation of elastic laminae of the arterial tunica media, contributing to aneurismal expansion [Shimizu *et al.* 2004]. Actually, cytokines secreted by both Th1 and Th2 cells are able to regulate MMP, serine protease, and cathepsin expressions. Indeed, it has been shown that depending on the particular experimental conditions, both Th1 and Th2 cytokines can induce or inhibit expression of specific MMPs [Shimizu *et al.* 2005]. In another more recent study in human aneurismal and atherosclerotic tissues, a set of cytokines and transcription factors involved in both Th1 and Th2 responses were assessed. The results of this study indicated that there was not a clear Th1/Th2 polarization in aneurismal tissue although the general inflammatory level was enhanced compared to atherosclerotic tissue [Lindeman *et al.* 2008]. Thus, it looks fairly clear that inflammatory disorders in aneurismal pathology are complicated and further studies would be helpful to better understand and clarify the whole mechanism involved in this fatal disease.

1.3.4. Oxidative stress

Regarding oxidative stress, there is evidence for increased levels of reactive oxygen species (ROS) and, consequently, an increased level of oxidative stress in human AAA [Miller,

Jr. *et al.* 2002]. The presence of oxidative stress contributes importantly to the pathophysiology of inflammation [McCormick *et al.* 2007]and so, to the development of AAA. Moreover, ROS seem to play a key role in regulation of MMP [Wassef *et al.* 2001]. Actually, it was shown in a $CaCl_2$ induced AAA model in mice that inhibition of ROS attenuated the expression of MMP-2 and MMP-9 in the aortic tissue, reducing aneurysm formation [Xiong *et al.* 2009].

1.3.5. Intraluminal thrombus

Another key player to take into account in biological mechanisms of aneurismal development is the intraluminal thrombus (ILT). The common presence of an ILT in AAA provides a permanent interface with the circulating blood. Neutrophils are one of the main cell types contained in the ILT. These cells circulate in blood and play an important role during the first stage of inflammatory response, contributing importantly to the recruitment, activation, and programming of antigen-presenting cells [Nathan 2006]. Neutrophils take part in signaling processes for the amplification of their own recruitment and for the attraction of monocytes and dendritic cells. They also influence whether macrophage differentiation follows a predominantly pro- or anti-inflammatory pattern [Chertov *et al.* 1997; Bennouna *et al.* 2003; Tsuda *et al.* 2004]. Neutrophil recruitment in ILT associated to human AAA seemed to be mediated by cytokines such as platelet-derived RANTES and neutrophil-derived IL-8 [Houard *et al.* 2009]. Interestingly, the content of MMP-9 was significantly increased in the ILT compared to the content of MMP-9 in the wall of human AAA samples, and neutrophils were shown to be responsible for this increased content of MMP-9 in the ILT [Fontaine *et al.* 2002]. Moreover, matrix-degrading protease expression and activity differs between thrombus free and thrombus covered wall of AAA [Kazi *et al.* 2005]. Indeed, upregulation of MMP expression was found in thrombus-free aneurysmal wall, however, only MMP-9 activity was increased at the interface between the thrombus and the underlying wall according with previous studies [Fontaine *et al.* 2002]. Furthermore, MMP-9 activity is regulated by neutrophil gelatinase associated lipocalin (NGAL), that forms a complex with MMP-9 protecting it from degradation [Yan *et al.* 2001]. A recent study showed that complexes of NGAL/MMP-9 were present in thrombus, and wall of human biopsies of AAA [Folkesson *et al.* 2007]. The same study also showed that neutrophils were the major source of NGAL expression. Moreover, neutrophils specifically release neutrophil collagenase, also called MMP-8, which is a type I collagenase. MMP-8 concentration was significantly higher in human AAA biopsies compared to normal infrarenal aortas [Wilson *et al.* 2005]. However, MMP-8 deficiency in mice did not help to diminish aortic dilation after elastase perfusion compared to wild-type mice, suggesting that

MMP-8 serves only as a marker for the presence of neutrophils and is not critical for AAA formation [Eliason *et al.* 2005]. Interestingly, depletion of circulating neutrophils in an elastase-induced model of AAA in mice inhibited AAA development through a non-MMP2/-9-mediated mechanism [Eliason *et al.* 2005]. The influence of ILT on MMP activity within the aortic wall needs to be further determined. A later study showed that mice with a loss of function on neutrophil recruitment did not develop AAA after elastase perfusion [Pagano *et al.* 2007]. Thus, it seems that thrombus formation and further neutrophil recruitment may be one of the triggering events involved in the origin of AAA. Indeed, it has been recently demonstrated that ILT releases plasma markers of platelet activation and further renewal that correlate with progression of AAA [Touat *et al.* 2006]. In the same paper, it was shown for the first time that inhibition of platelet aggregation pacified thrombus activity and attenuated aneurismal enlargement in a rat model of AAA. These results were later confirmed in another study of the same research group in which platelet activation was blocked by using an antagonist of $P2Y_{12}$ receptor in a rat model of AAA [Dai *et al.* 2009]. Also, ILT seems to have a biomechanical influence during aneurysmal enlargement. The presence of ILT reduced peak wall stress in computed models of AAA [Georgakarakos *et al.* 2009]. Moreover, for aneurysms between 5 and 7 cm, a strong correlation between the relative volume of ILT and the degree of peak wall stress reduction was found. However, the hypothesis that ILT might play a key role in development and evolution of AAA has gone less noticed than the hypothesis focusing on the influence of MMPs and inflammation, but it seems clear that further studies are needed to better determine the relevance and implications of ILT in aneurismal formation as well as its possible use in AAA treatment.

Taken all together, the molecular mechanisms of AAA could be summarized as the generation of biomechanical wall stress: First, ECM degradation in the media layer of the aortic wall produces loss of elastic capacity; and second, atherosclerotic plaque formation produces fragility, leading both together to aneurismal rupture.

1.4. Inflammation in AAA vs. atherosclerosis

Although the presence of atherosclerosis is closely related to AAA and the traditional belief is that atherosclerosis is an active component of aneurismal degeneration, there is a controversial debate about the etiologic relationship between both types of lesions, their mechanisms, and the contribution of atherosclerosis to the aneurismal enlargement. Recruitment of inflammatory cells can be observed in both types of lesions, however, the localization of inflammatory cells within the aortic walls differs between them (Figure 2). In atherosclerosis, infiltration of macrophages produces inflammation in the tunica subintima;

whereas infiltrated macrophages in AAA are rather in the media and adventitia layers of the aortic wall [Diehm *et al.* 2007].

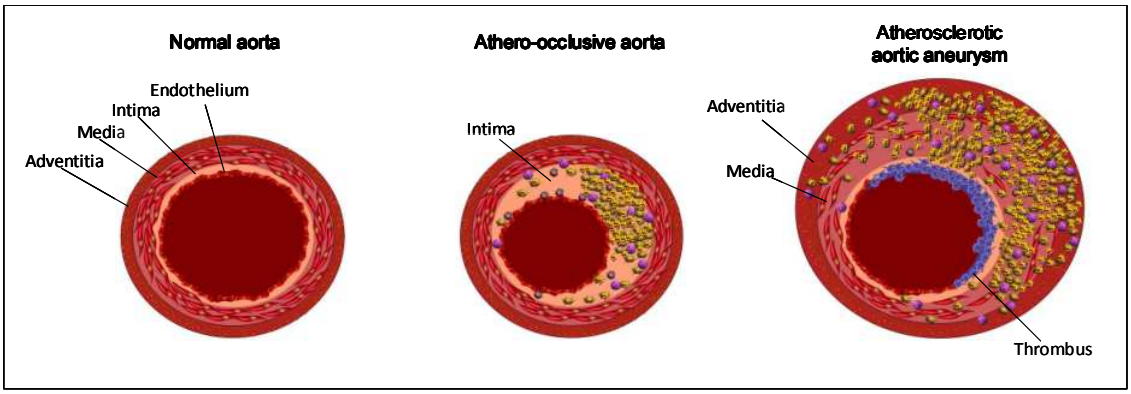

Figure 2. Localization of inflammatory macrophages in atherosclerosis and in AAA.

Furthermore, some studies support the hypothesis that immune response in both pathologies is different. It is fairly clear that atherosclerosis has a predominant Th1 type immune response [Hansson *et al.* 2006], but in AAA studies the results were not so conclusive. Some studies demonstrated that there is a predominant Th2 response in aneurismal tissue as opposed to a predominant Th1 response in atherosclerotic lesions [Schonbeck *et al.* 2002] while others supported the hypothesis that Th1 immune responses prevail in aneurismal lesions [Galle *et al.* 2005; Middleton *et al.* 2007]. Further investigations are needed to clarify and establish the similarities and differences of inflammation and immune responses between athero-occlusive aortic and aneurismal disease. However, robust data does indicate that diabetes is inversely associated with the presence of AAA whereas it is directly associated with atherosclerotic pathology [Lederle *et al.* 1997a; Wanhainen *et al.* 2005; Golledge *et al.* 2006].

1.5. Abdominal aortic aneurysms vs. thoracic aortic aneurysms

Both abdominal and thoracic aortic aneurismal diseases (Figure 3) consist of the degradation of the ECM at the aortic media layer with a consistent weakening of the aortic wall. This wall weakening together with biomechanical wall stress can produce rupture of the aortic wall and further hemorrhage, resulting in rapid mortality in most cases. From the epidemiologic point of view, the frequency of AAA is more than three times higher than that of TAA and dissection aneurysms [Sakalihasan *et al.* 2005; Griepp *et al.* 1999], and although the clinical consequences of both pathologies are very similar, the mechanisms involved in are quite different.

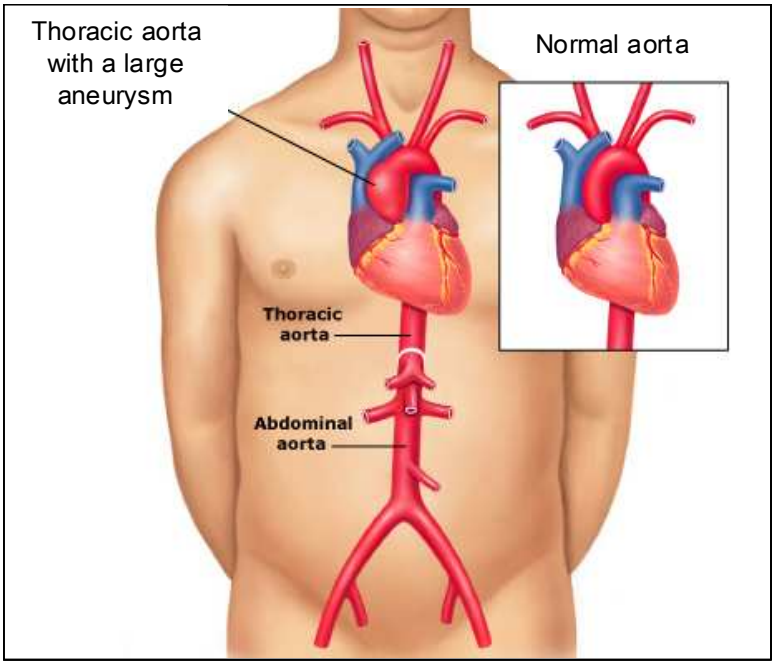

Figure 3. Localization of thoracic aneurismal aorta.

The main differences between the two pathologies were recently described by Michel *et al.* [Michel *et al.* 2008] as follows:

a) Vascular smooth muscle cells pathology is secondary in AAA and primary in TAA.

b) AAA is linked to atherothrombosis whereas TAA is not.

c) AAA is considered as polygenic whereas TAA is mainly monogenic disease.

d) Age and gender trends are different.

e) An intra-luminal thrombus is frequently present in AAA but absent in TAA.

f) Inflammatory processes play an important role in AAA but not in TAA.

Thus, it is clear that aneurysms in these two locations are the consequences of different biological processes and should not be mistaken for each other. The understanding of how remodeling is produced in both diseases is of great importance for their specific treatment.

1.6. Symptoms, detection and treatments

Unfortunately, the vast majority of AAA's are asymptomatic and diagnosed during an abdominal examination for other reasons. Abdominal palpation accompanied by ultrasound methods like echography can be used for diagnosis of AAA. However these techniques are still expensive to be used in a systematic screening of the population at risk [Cosford *et al.* 2007].

Few patients present clinical symptoms of AAA including low back pain, flank pain, abdominal pain, pulsating abdominal mass [Upchurch, Jr. *et al.* 2006]. In case of rupture, clinical manifestations presented as a consequence of the internal hemorrhage include: low back pain, flank pain, abdominal pain and hypovolemic shock [Upchurch, Jr. *et al.* 2006] (i.e. hypotension, tachycardia, cyanosis, and altered mental status). However, diagnosis is normally not possible prior to rupture due to the traditionally asymptomatic character of AAA [Ailawadi *et al.* 2003].

Nevertheless, in case of detection, rupture can be prevented by vascular surgery which decreases mortality in AAA patients. The two primary surgeries applied in order to repair AAA are open and endovascular. Open aortic repair (OAR) has been the standard care for more than 50 years and involves direct access to the aorta through an incision in the abdomen. This repair method is well established as definitive and it does not require follow-up radiologic studies. However, endovascular aortic repair (EVAR) was introduced in the early 1990s and consists of placing a stent exoskeleton within the lumen of AAA, decreasing the pressure supported by the aortic wall and leading to reduction of aneurismal size. In this case, the access to the lumen of the abdominal aorta during surgery is made via small incisions over the femoral vessels. It seems that elective EVAR has replaced OAR as the most common method of AAA repair in countries like the United States. Interestingly, EVAR can be adopted in elderly surgical patients, aged more than 85 years, who can not go through AOR intervention. Studies have been shown that in cases in which AAA ruptures, the use of EVAR as surgery method has a lower postoperative mortality rate than AOR [Giles *et al.* 2009; McPhee *et al.* 2009]. Despite the observed decrease in mortality after the application of EVAR for large or ruptured aneurysms, survival is not improved by elective repair of abdominal aortic aneurysms smaller than 5.5 cm [Lederle *et al.* 2002]. In 2003, Brewster et al. [Brewster *et al.* 2003] set out the following list of recommendations for AAA repair:

a) The arbitrary setting of a single threshold diameter for elective AAA repair applicable to all patients is not appropriate, as the decision for repair must be individualized in each case.

b) Randomized trials have shown that the risk of rupture of small (<5 cm) AAA is quite low, and that a policy of careful surveillance up to a diameter of 5.5 cm is safe, unless rapid expansion (>1 cm/y) or symptoms develop. However, early surgery is comparable to surveillance with later surgery, so patient preference is important, especially for AAA 4.5 cm to 5.5 cm in diameter.

c) Based upon the best available current evidence, a diameter of 5.5 cm appears to be an appropriate threshold for repair in an "average" patient. However, subsets of younger low-risk patients, with long projected life expectancy, may prefer early repair. If the surgeon's personal documented operative mortality rate is low, repair may be recommended at smaller sizes (4.5-5.5 cm) if that is the patient's preference.

d) For women, or AAA with greater than average rupture risk, elective repair at 4.5 cm to 5.0 cm is an appropriate threshold for repair.

e) For high-risk patients, delaying repair until the diameter larger may be warranted, especially if EVAR is not possible.

f) In view of its uncertain long-term durability and effectiveness, as well as the increased surveillance burden, EVAR is most appropriate for patients at increased risk for conventional open aneurysm repair.

g) EVAR may be the preferred treatment method for older, high-risk patients, those with "hostile" abdomens, or other clinical circumstances likely to increase the risk of conventional open repair, if their anatomy is appropriate.

h) Use of EVAR in patients with unsuitable anatomy markedly increases the risk of adverse outcomes, need for conversion to open repair, or AAA rupture.

i) At present, there does not appear to be any justification that EVAR should change the accepted size thresholds for intervention in most patients.

j) In choosing between open repair and EVAR, patient preference is of great importance. It is essential that the patients be well informed when making such choices.

This guideline of AAA treatment is totally based on the diameter of AAA. Indeed, diameter is the single most important factor deciding whether to repair an aneurysm or to monitor it by ultrasound techniques [Ouriel 2009] and up to date, surgery is the only available treatment of AAA [Golledge *et al.* 2009]. There is a big effort to reduce the cost of both diagnosis and treatment methods of AAA.

1.7. Necessity of biomarkers

Ideally, non-invasive diagnostic methods for AAA would be available to physicians in routine screening of at risk population. Serum/plasma samples, being readily obtainable in

most examination settings, provide a readily available biofluid from which to make such diagnosis. Unfortunately, as for most diseases, no accurate, objective biomarkers exist. During the last two years, four scientific reviews tried to summarize all the potential biological markers for AAA. These reviews point to the importance of developing disease specific biomarkers and specifically for diagnosis and follow-up of AAA. The two reviews by Hellenthal *et al*. [Hellenthal *et al.* 2009a; Hellenthal *et al.* 2009b]assessed separately ECM degeneration and inflammation factors as potential biomarkers for AAA progression, while Urbonavicius *et al*. [Urbonavicius *et al.* 2008], and Golledge *et al*. [Golledge *et al.* 2008b] focused in all possible disorders implicated in AAA: lipids, thrombus related proteins, ECM degradation, inflammation and triggering factors. The principal studies described in these reviews are summarized in Table 1 and Table 2. Table 1 shows the relationship between the expression levels of potential biological markers and the presence of AAA. Table 2 shows the potential biological markers and the correlation between their expression levels and aneurismal development.

Table 1. Potential biomarkers for AAA detection

Potential biomarker	Number of patients in the study (Cases /Controls)	Association between biomarker concentration and AAA presence	Reference
PROTEINS INVOLVED IN EXTRACELLULAR MATRIX DEGENERATION			
PIIINP	95/83	no difference	[Eugster *et al.* 2005]
	201/246	no difference	[Wilmink *et al.* 2002]
	86/20	significantly increased in cases	[Treska *et al.* 2000a]
	87/90	significantly increased in cases	[Satta *et al.* 1995]
MMP-9	53/26	significantly increased in cases	[Watanabe *et al.* 2006]
	95/83	no difference	[Eugster *et al.* 2005]
	22/12	no difference	[van Laake *et al.* 2005]
	45/10	significantly increased in cases	[Sangiorgi *et al.* 2001]
	25/20	significantly increased in cases	[Hovsepian *et al.* 2000]
	22/17	significantly increased in cases	[McMillan *et al.* 1999]
PROTEINS ASSOCIATED WITH THROMBOSIS			
Tissue plasminogen activator	40/41	no difference	[Skagius *et al.* 2008]
	42/100	significantly increased in cases	[Wanhainen *et al.* 2007]
	89/98	no difference	[Fowkes *et al.* 2006]
	23/20	no difference	[Holmberg *et al.* 1999]
	40/200	no difference	[Lee *et al.* 1996]
D-dimer	40/41	significantly increased in cases	[Skagius *et al.* 2008]
	89/98	significantly increased in cases	[Fowkes *et al.* 2006]
	18/10	significantly increased in cases	[Serino *et al.* 2002]

	36/25	significantly increased in cases	[Yamazumi *et al.* 1998]
	40/200	significantly increased in cases	[Lee *et al.* 1996]
	41/30	significantly increased in cases	[Aramoto *et al.* 1994]
Fibrinogen	110/110	significantly increased in cases	[Al Barjas *et al.* 2006]
	89/98	significantly increased in cases	[Fowkes *et al.* 2006]
	36/68	no difference	[Spring *et al.* 2006]
	337/6049	significantly increased in cases	[Singh *et al.* 2001]
	23/20	significantly increased in cases	[Holmberg *et al.* 1999]
	21/84	no difference	[Blann *et al.* 1998]
	40/200	significantly increased in cases	[Lee *et al.* 1996]
	22/244	no difference	[Franks *et al.* 1996]
PROTEINS INVOLVED IN INFLAMMATION PROCESSES			
Interleukin-6	27/15	significantly increased in cases	[Dawson *et al.* 2007]
	89/98	significantly increased in cases	[Fowkes *et al.* 2006]
	74/30	significantly increased in cases	[Treska *et al.* 2000b]
	50/80	significantly increased in cases	[Juvonen *et al.* 1997]
LIPIDS			
Lipoprotein a	425/492	no difference	[Jones *et al.* 2007]
	438/438	significantly increased in cases	[Sofi *et al.* 2005]
	75/43	significantly increased in cases	[Schillinger *et al.* 2002]
	29/274	significantly increased in cases	[Papagrigorakis *et al.* 1997]
	22/244	no difference	[Franks *et al.* 1996]
	69/1460	no difference	[Simoni *et al.* 1996]
High-density lipoprotein	30/26	significantly decreased in cases	[Rizzo *et al.* 2009]
	35/140	significantly decreased in cases	[Wanhainen *et al.* 2005]
	206/252	no difference	[Hobbs *et al.* 2003]
	337/6049	significantly decreased in cases	[Singh *et al.* 2001]
	25/266	no difference	[Naydeck *et al.* 1999]
	21/84	significantly decreased in cases	[Blann *et al.* 1998]
	69/1460	significantly decreased in cases	[Simoni *et al.* 1996]
	114/57	significantly increased in cases	[Louwrens *et al.* 1993]
Low-density lipoprotein	30/26	no difference	[Rizzo *et al.* 2009]
	35/140	no difference	[Wanhainen *et al.* 2005]
	206/252	significantly increased in cases	[Hobbs *et al.* 2003]
	337/6049	no difference	[Singh *et al.* 2001]
	25/266	significantly increased in cases	[Naydeck *et al.* 1999]
	21/84	no difference	[Blann *et al.* 1998]
	69/1460	no difference	[Simoni *et al.* 1996]
	114/57	no difference	[Louwrens *et al.* 1993]

PIIINP: aminoterminal propeptide of type III procollagen; MMP-9: matrix metalloproteinase 9. Presented data were adapted from: [Hellenthal *et al.* 2009a; Hellenthal *et al.* 2009b; Urbonavicius *et al.* 2008; Golledge *et al.* 2008b].

Biological markers assessed for AAA detection can be classified in four categories: proteins involved in extracellular matrix degeneration (aminoterminal propeptide of type III procollagen (PIIINP) and matrix metalloproteinase 9), proteins associated with thrombosis (tissue plasminogen activator, D-dimer, and fibrinogen), proteins involved in inflammatory processes (interleukin-6), and lipids (lipoprotein a, high-densitity lipoprotein, and low-density lipoprotein). For all described potential biomarkers, studies showed either significant difference or no differences in biomarker plasma level between AAA and control patients independently on the sample size of the studied population, except for D-dimer, that was significantly increased in AAA plasma samples of all performed studies.

Table 2. Potential biomarkers for AAA progression

Potential biomarker	Number of patients in the study	Correlation between biomarker expression and AAA progression	Reference
POTENTIAL BIOMARKERS INVOLVED IN EXTRACELLULAR MATRIX DEGENERATION			
Serum elastin peptides	36	significant correlation	[Lindholt *et al.* 2000]
	79	significant correlation	[Lindholt *et al.* 1997]
	112	correlation, no p value available	[Lindholt *et al.* 2001c]
	70	correlation, no p value available	[Lindholt *et al.* 2001d]
Serum elastin derived peptides	60	no correlation	[Petersen *et al.* 2001]
PIIINP	36	no correlation	[Lindholt *et al.* 2000]
	112	correlation, no p value available	[Lindholt *et al.* 2001c]
	50	significant correlation	[Juvonen *et al.* 1997]
	55	significant correlation	[Satta *et al.* 1997]
	86	no correlation	[Treska *et al.* 2000a]
PICP	55	no correlation	[Satta *et al.* 1997]
	86	no correlation	[Treska *et al.* 2000a]
Elastase- α-1-antitrypsin complexes	36	no correlation	[Lindholt *et al.* 2000]
	79	significant correlation	[Lindholt *et al.* 2003a]
α-1-antitrypsin	35	significant correlation	[Vega *et al.* 2009]

	36	significant correlation	[Lindholt *et al.* 2000]
MMP-2	36	no correlation	[Lindholt *et al.* 2000]
	76	no correlation	[Eugster *et al.* 2005]
MMP-9	36	significant correlation	[Lindholt *et al.* 2000]
	208	no correlation	[Karlsson *et al.* 2009]
	76	no correlation	[Eugster *et al.* 2005]
TIMP-1	36	no correlation	[Lindholt *et al.* 2000]
TIMP-2	36	no correlation	[Lindholt *et al.* 2000]
POTENTIAL BIOMARKERS ASSOCIATED WITH THROMBOSIS			
Cystatin C	142	significant inverse correlation	[Lindholt *et al.* 2001b]
	8	significant inverse correlation	[Shi *et al.* 1999]
Plasmin- antiplasmin complexes	70	significant correlation	[Lindholt *et al.* 2001d]
Plasminogen	70	no correlation	[Lindholt *et al.* 2001d]
Urokinase plasminogen activator	70	no correlation	[Lindholt *et al.* 2003b]
Tissue plasminogen activator	70	significant correlation	[Lindholt *et al.* 2003b]
Plasminogen activator inhibitor-1	70	no correlation	[Lindholt *et al.* 2003b]
APC-PCI	168	no correlation	[Kolbel *et al.* 2008]
D-dimer	36	significant correlation	[Yamazumi *et al.* 1998]
Fibrinogen/fibrin	36	significant correlation	[Yamazumi *et al.* 1998]
Fibrinogen	110	significant correlation	[Al Barjas *et al.* 2006]
POTENTIAL BIOMARKERS INVOLVED IN INFLAMMATION PROCESSES			
IFN-gamma	50	significant correlation	[Juvonen *et al.* 1997]
TNF	50	no correlation	[Juvonen *et al.* 1997]
TNF-alpha	90	significant correlation	[Treska *et al.* 2000a]
TGF-beta 1	70	no correlation	[Pan *et al.* 2003]
Macrophage migration inhibitory factor	70	no correlation	[Pan *et al.* 2003]
	98	significant correlation	[Pan *et al.* 2003]
Osteopontin	198	significant correlation	[Golledge *et al.* 2007b]
Osteoprotegerin	146	significant correlation	[Moran *et al.* 2005]
Interleukin-1 beta	50	no correlation	[Juvonen *et al.* 1997]
Interleukin-6	50	no correlation	[Juvonen *et al.* 1997]
	200	no correlation	[Karlsson *et al.* 2009]

	7	significant correlation	[Rohde *et al.* 1999]
Interleukin-8	90	significant correlation	[Treska *et al.* 2000b]
Cotinine	79	significant correlation	[Lindholt *et al.* 2003a]
	210	no correlation	[Wilmink *et al.* 1999]
	70	significant correlation	[Lindholt *et al.* 2003a]
Homocysteine	108	significant correlation	[Halazun *et al.* 2007]
	70	no correlation	[Lindholt *et al.* 2003b]
	58	significant correlation	[Brunelli *et al.* 2000]
C reactive protein	114	no correlation	[Domanovits *et al.* 2002]
	151	no correlation	[Lindholt *et al.* 2001b]
	545	significant correlation	[Norman *et al.* 2004]
Serum highly sensitive C reactive protein	39	significant correlation	[Vainas *et al.* 2003]
INFECTIOUS TRIGGERING FACTORS			
IgA-CP	70	significant correlation	[Lindholt *et al.* 2003a]
Chlamidophila pneumoniae infection	110	significant correlation	[Lindholt *et al.* 2001a]
Chlamidophila pneumoniae serology	68	significant correlation	[Falkensammer *et al.* 2007]
	119	no correlation	[Nyberg *et al.* 2007]

PIIINP: aminoterminal propeptide of type III procollagen; PICP: carboxyterminal propeptide of type I procollagen; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of matrix metalloproteinases; APC-PCI: activated protein C-protein C inhibitor complex; IFN: interferon; TNF: tumor necrosis factor; TGF: transforming growth factor; IgA-CP: immunoglobulins against Chlamidophila pneumonia. Presented data were adapted from: [Hellenthal *et al.* 2009a; Hellenthal *et al.* 2009b; Urbonavicius *et al.* 2008; Golledge *et al.* 2008b]

Biological markers assessed for AAA progression can be classified in four categories: biomarkers involved in extracellular matrix degeneration (serum elastin peptides, serum elastin derived peptides, aminoterminal propeptide of type III procollagen (PIIINP), elastase-alpha-1-antitrypsin complexes, alpha-1 antitrypsin, MMP-2, MMP-9, TIMP-1, and TIMP-2), biomarkers associated with thrombosis (cystatin C, plasmin-antiplasmin complexes, plasminogen, urokinase plasminogen activator, tissue plasminogen activator, plasminogen activator inhibitor-1, APC-PCI, D-dimer, fibrin to fibrinogen ratio, and fibrinogen), biomarkers involved in inflammatory processes (interferon-gamma, tumor necrosis factor, tumor necrosis factor-alpha, transforming growth factor-beta 1, macrophage migration inhibitory factor, osteopontin, osteoprotegerin, interleukin-1 beta, interleukin-6, interleukin-8, cotinine,

homocysteine, C reactive protein, and serum highly sensitive C reactive protein), and infectious triggering factors (immunoglobulins against Chlamidophila pneumonia, Chlamidophila pneumonia infection, and Chlamidophila pneumonia serology). For most of described potential biomarkers, studies showed either significant correlation or no correlation between the level of biomarker in plasma and AAA expansion independently on the sample size of the studied population.

It seems clear that described data on biomarkers for AAA is not conclusive and more epidemiological studies on large multicenter cohorts are needed. All described potential biomarkers were probably evaluated because of their role in biological mechanisms involved specifically in AAA or generally in cardiovascular pathology. However, another attractive alternative way to assess biomarker discovery for AAA may be through high-throughput analysis of biomolecules in the absence of any *a priori* hypothesis. This kind of studies could lead to the identification of new candidates as well as a better comprehension of mechanisms involved in aneurismal pathology.

2. How to address the search for AAA biological markers

From a clinical point of view, biomarkers are intended to provide attending physicians with objective biochemical markers of acute or chronic medical conditions so that they may better manage patient status. Thus, biomarkers can be classified into three groups according to the stage of disease. First, as screening biomarkers, when detecting disease in asymptomatic patients; then, as diagnostic biomarkers, when patients are suspected to have disease; and finally, as prognostic biomarkers, when disease is already established and evolution is followed. Currently, diagnostic and prognostic cardiovascular biomarkers are available while screening biomarkers are not yet widely described [Gerszten *et al.* 2008]. Regarding AAA, screening biomarkers are important from a preventive perspective. They allow detection of patients at potential risk to develop aneurismal disease; ideally, in a very early stage of aneurismal formation or progression with further implications on treatment and follow-up. Five phases of screening biomarker development for early detection of cancer were described in detail by Pepe *et al.* [Pepe *et al.* 2001]. These phases go from bench to bedside and consider all general aspects in research and clinics during biomarker discovery. Later, they were adapted to cardiovascular biomarker development by Ramachandran S. Vasan [Vasan 2006], who also described the molecular basis and practical considerations to take into account during this process. Table 3 presents a summary of the main considerations to take into account when going through the five phases of biomarker development.

Table 3. Five phases of biomarker development

Phase	Description
Phase 1: Discovery phase	Exploratory studies allow identification of potential biomarkers.
Phase 2: Validation phase	Clinical studies evaluate capacity of the biomarker to discriminate between healthy and diseased patients.
Phase 3: Preclinical phase	Clinical studies evaluate capacity of the biomarker to detect disease before it becomes clinical, and a "screen positive" rule is defined.
Phase 4: Prospective phase	Prospective screening studies are conducted to establish specificity and sensitivity of the biomarker
Phase 5: Impact phase	Large scale studies assess impact of screening on reducing mortality.

Adapted from [Pepe *et al.* 2001] and [Nordon *et al.* 2009].

Most current cardiovascular biological markers are implicated in pathways related to atherosclerotic cardiovascular diseases, such as inflammation or cholesterol biosynthesis [Gerszten *et al.* 2008]. Consistently, high sensitivity, specificity, and predictive values are of great importance for AAA screening biomarkers and atherosclerotic biomarkers should just be used as complementary information since most of the aneurismal patients suffer from atherosclerosis but most of the atherosclerotic patients do not develop AAA. Nevertheless, one should keep in mind that an ideal single biomarker does not exist for every disease [Mischak *et al.* 2007] and this is logical since, usually, more than one biomolecule (gene, protein and/or metabolite) are implicated in the pathophysiology diseases.

2.1. Choice of adequate techniques

2.1.1. Representative biomolecules of diseased states

The knowledge of the DNA sequence is essential for the understanding of pathologies. However, characterization of the final products of gene expression, proteins, allows for a more meaningful comprehension of disease phenotype. Indeed, the complete sequencing of the human genome showed that fewer than 30,000 genes encode for more than a million proteins [Venter *et al.* 2001]. Thus, after alternative mRNA splicing and post-translational modifications (PTMs), one gene does not code for one protein but for many, increasing considerably functional diversity (Figure 4). Actually, all this variety of gene expression ends up in specific phenotypes of cells, organs and tissues, which are finally defined by an instantaneous proteomic profile.

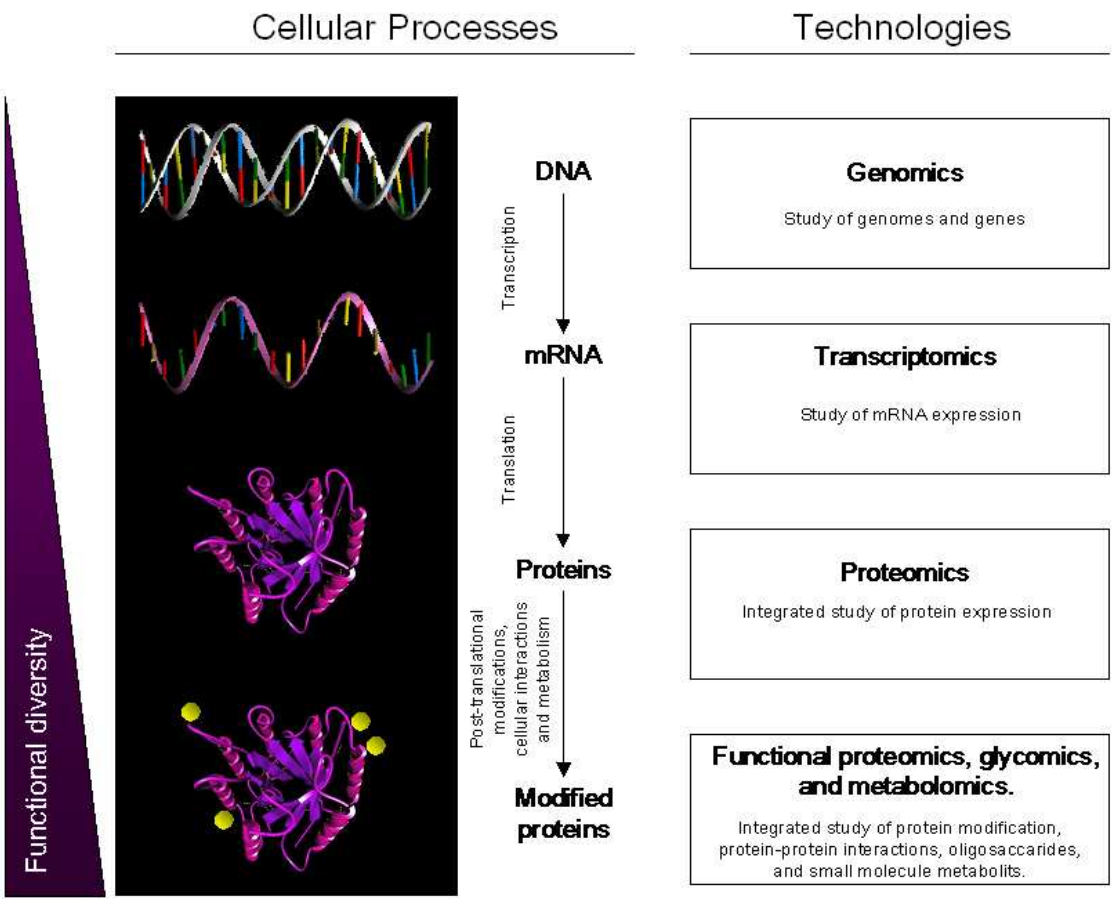

Figure 4. Overview of cellular processes and modern technologies available for their study.

Proteins are involved in every cellular function, they control every regulatory mechanism and they are, as cause or effect, modified in disease states [Arrell *et al.* 2001]. As a consequence, a proteome is defined as a dynamic system that involves changing both protein expression levels and PTMs over time in response to different stimuli. Indeed, in order to become active, many proteins need to go through PTMs such us phosphorylation, glycation or methylation. Proteolytic processing is also important since most of the time an inactive protein or enzyme only can become active after the proteolytic cleavage of the protein precursor, also called proprotein or proenzyme. In other cases, the N-terminal peptide of a protein works as a signal peptide that directs the transport into a specific organelle in the cell. Once the protein is transported, the signal peptide is cleaved by specific proteolytic enzymes. Identifying changes in both the expression and modification of proteins in a disease state compared to a disease-free control offers direct insight into the disease process [Wang *et al.* 2008]. In the particular case of aneurysmal disease, proteolysis is one of the major processes involved in the evolution of the pathology, so peptides resulting from this proteolytic activity are interesting to follow and to better understand the progressive degradation of the aortic wall as it was recently showed by Dejouvencel *et al*. [Dejouvencel *et al.* 2009].

To this end, it seems clear that proteins might be ideal biological markers to be used for screening, diagnosis and prognosis of any human disease, and proteomic technology, more specifically differential proteomic analysis, seems to be challenged to be the technique of choice in the discovery of these biological markers.

2.1.2. Application to clinical studies

The application of proteomics to the study of human diseases and translation of this technology to the clinic has generated a new emerging field called "Clinical proteomics" [Beretta 2007]. Thus, clinical proteomics has focused on the discovery of disease biomarkers as well as of novel drug targets. Indeed, many authors agreed that proteomic tools are designed to address the question of cardiovascular biomarker discovery [Anderson 2005a; Mayr *et al.* 2006; Edwards *et al.* 2008; Nordon *et al.* 2009; Moxon *et al.* 2009]. Collecting biological samples of patients recruited into clinical protocols is essential in clinical proteomics. Most of the time, these are long-term studies and cell sample, cell culture, protein extraction and protein storage are all key steps that must be performed the same way throughout the study. A guideline to follow when designing and performing a clinical proteomic project was described by Mischack et al. [Mischak *et al.* 2007] and is summarized below:

a) Define clear clinical question and how the outcome of the study would improve the diagnosis and/or treatment of the disease.

b) Define the patient and control populations, clinical data to be collected, as well as protocols for sampling and sample preparation.

c) Define the types of samples needed for the discovery and validation phases.

d) Define and validate the analytical platforms for discovery (those for validation may well differ).

e) Obtain institutional review board approval and written informed consent from the participant.

f) Perform a pilot study on a validated discovery platform.

g) Statistically evaluate data from the pilot study to calculate the number of cases and controls for the training set.

h) Perform study of the training set on the validated platform based on the calculated number of cases and controls.

i) Evaluate findings from the training set of blinded samples.

j) Deposit datasets in a public database.

k) Using these results, transfer the assay to the application platform and test using training (if applicable) and subsequently a blinded set.

l) Apply towards clinical use to show whether the findings improve the current clinical situation.

Proteomic strategy for biomarker discovery is based on differential proteomic analysis. In general, differential proteomics is the comparison of protein profiles from various samples obtained in different conditions in order to identify differences in protein abundance without any *a priori* hypothesis [Cieniewski-Bernard *et al.* 2008] and this last statement is one of the strong points of differential proteomic analysis. Congruently, search of biomarkers will be done comparing the proteome at disease state to the proteome at disease-free state. Comparison of different proteomic profiles can be achieved by gel-based and by gel-free methods, but in both cases, the use of mass spectrometry (MS) is essential for identification, and sometimes characterization of proteins.

2.1.3. Gel-based methods

The first proteomic methods used to analyze complex mixtures of proteins were gel-based methods. They consist of protein separation of each sample by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE); in which proteins are separated firstly; according to their isoelectric point (*pI*), and secondly; according to their molecular weight (*Mr*). The process of protein staining can be performed either before electrophoresis, when proteins are chemically modified with fluorescent dyes, or after electrophoresis. Chemical modification of proteins is strongly recommended when samples are scarce due to the high sensitivity of this type of labeling. Figure 5 presents possible staining methods used to compare protein expression profiles as well as PTM in 2D-gels.

Stained gels are scanned and images from diseased samples are compared to those of control samples using specifically designed software. Statistical evaluation of normalized volumes of spots reveals differentially expressed proteins that can be further identified by mass spectrometry (Figure 6).

Post-electrophoretic methods

Detection by colorimetry

Staining method	Detection Limit
Coomassie-R	8-100 ng
Coomassie-G Colloidal	8-100 ng
Silver nitrate (alkaline methods)	<1-10 ng
Zinc imidazole	1-10 ng
Silver nitrate (acidic methods)	1-5 ng

Fluorescent detection

Staining method	Detection Limit
SYPRO®-Orange, Red, Tangerine	4-30 ng
SYPRO®-Ruby	1 ng
Epicoccone (Lightning Fast®, Deep Purple®)	<1 ng

Pre-electrophoretic methods

Fluorescent detection

Staining method	Detection Limit
FlaSH$^{Pro®}$ Dyes	2-3 ng
DIGE-minimal labeling	0.1-2 ng
DIGE-saturation labeling	0.005-0.01 ng

Figure 5. Different staining methods for protein detection in 2D-PAGE. Adapted from ref [Miller *et al.* 2006].

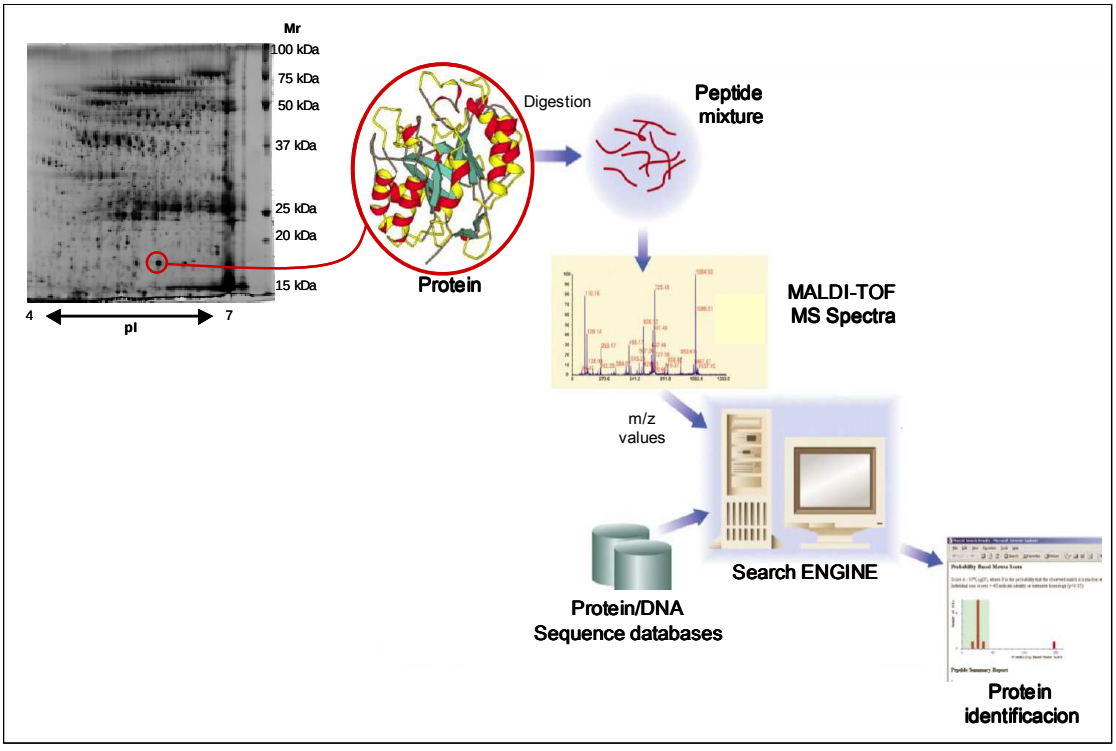

Figure 6. Typical workflow for protein identification when applying 2D-gel based proteomic analysis. Differentially expressed spots are excised from a preparative 2D-gel. Then, in-gel digestion is performed

and peptide mixture is analyzed by MALDI-TOF MS. m/z values of the MS spectra are compared against sequence databases to identify the protein contained in the spot (Adapted from MATRIX SCIENCE).

2.1.4. Gel-free methods

Regarding MS-based quantitative methods, they can be classified into two groups: isotopic labeling methods and label-free methods. Isotopic labeling requires chemical derivatization of proteins or peptides from different samples with tags that have the same molecular structure but a different isotopic composition. This allows the peptides of the same protein from different samples to have the same physico-chemical characteristics while having a different m/z value and thus generating a different signal in the spectra. In some cases the isotopic differences are not observed at the MS stage but at the MS/MS stage. In this case peptides with different tags have the same m/z value, i.e. they are isobaric, but during fragmentation the backbone of the tags also brakes and generates a specific m/z pattern which can be used to determine the differential protein expression between the samples. Figure 7 shows the different MS-based methods for quantification of proteins. Label free methods do not require any chemical modification of the samples before MS analysis and experimental values like peak intensity, peak area or the number of spectra corresponding to the identification of a specific protein, are used to determine differences in protein expression between samples.

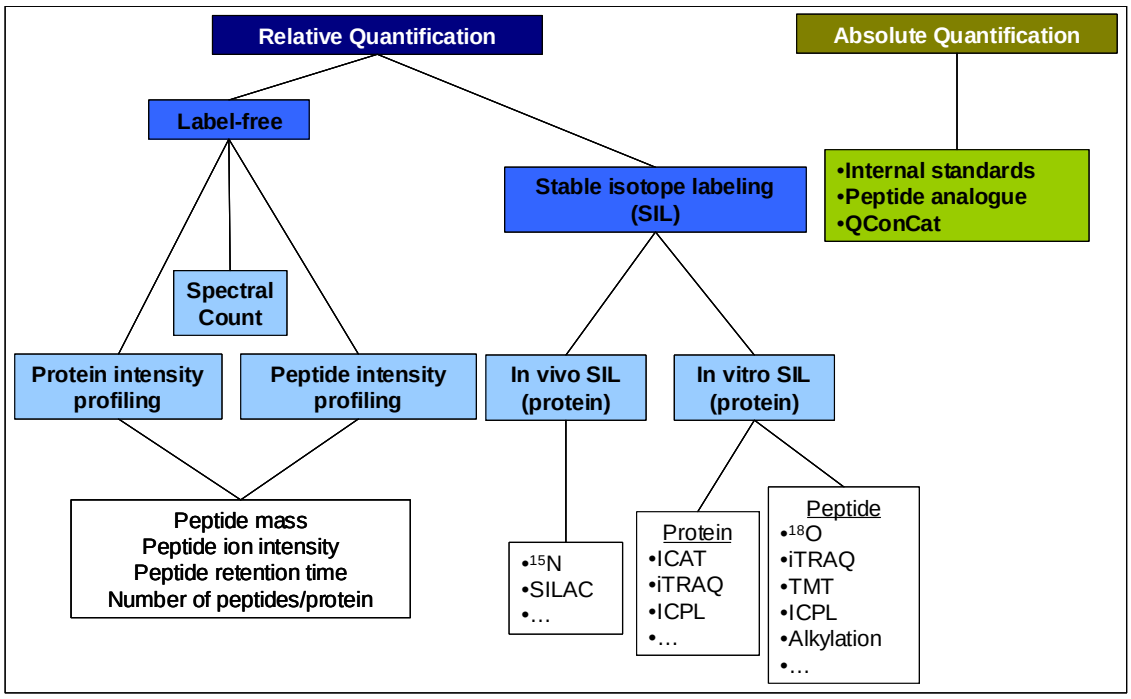

Figure 7. Different mass spectrometry based methods for relative and absolute quantification of proteins by differential proteomic analysis.

2.1.5. Mass spectrometry

Mass spectrometry is an analytical technique that uses the measurement of mass-to-charge ratios (m/z) of proteins and peptides to determine differences on protein expression between samples. The basis of mass spectrometry is founded on the ionization of a sample to generate charged species, analytes, which depending on their m/z ratio will have different electric and magnetic behaviors. These electromagnetic characteristics are responsible for detection of each analyte that is represented by peaks in graph of intensity vs. m/z called spectra. Thus, all mass spectrometers have two main components: the ion source, where sample will be ionized, and the mass analyzer, where generated ions will be separated according to their m/z value. Figure 8 shows a scheme of principal components of a mass spectrometer. Different combinations of ion sources with mass analyzers give rise to different types of instruments. Most of the time, protein samples are digested into peptides before MS analysis, and comparison of experimental m/z values of digested peptides against theoretical data bases allows identification of proteins.

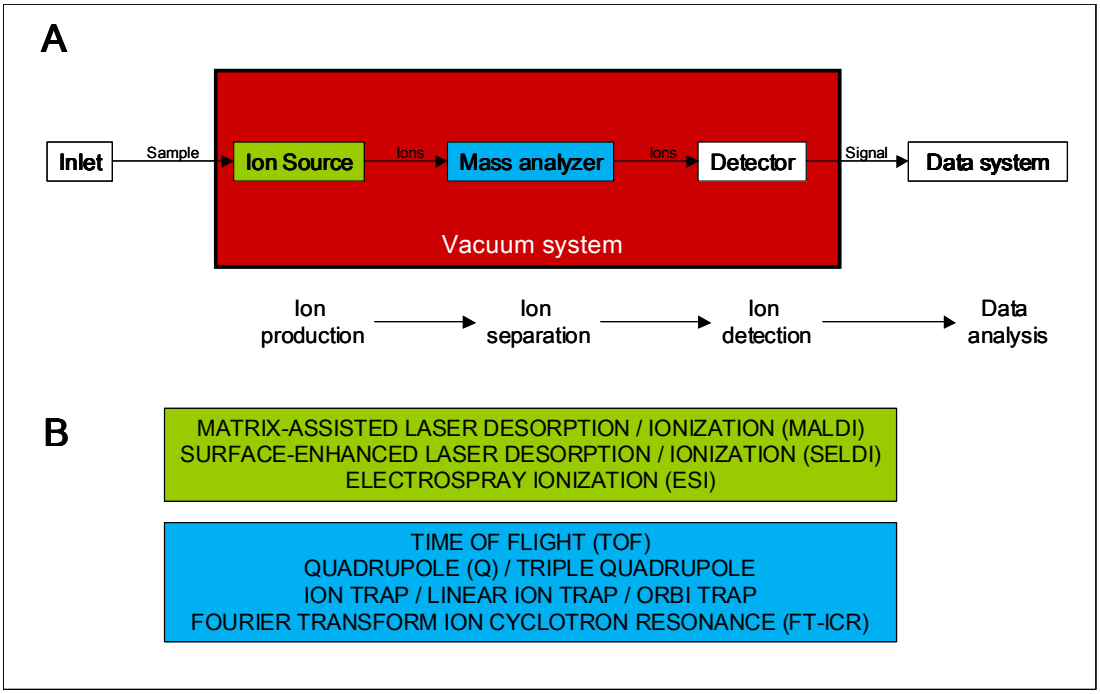

Figure 8. A: Different components of a mass spectrometer. B: Types of ion sources and mass analyzers used in proteomics.

Protein sequence information can also be achieved by tandem mass or MS/MS analysis, in which a specific m/z value, or a peptide, is selectively sent to a collision chamber. There, fragmentation is induced by collision with an inert gas and the m/z values of generated fragments are measured (Figure 9A). Fragment ions can be generated by different

mechanisms, e.g. collision induced / activated fragmentation (CID /CAD) and electron transfer dissociation (ETD). Fragmentation of peptide backbone is highly predictable and generated ions can be named following a nomenclature proposed by Roepstorff and Fohlman [Roepstorff *et al.* 1984] (Figure 9B). Thus, information of the sequence can be obtained by comparison against theoretical data bases. CID fragmentation predominantely produces *b* and *y* ions while ETD fragmentation produces mainly *c* and *z* ions.

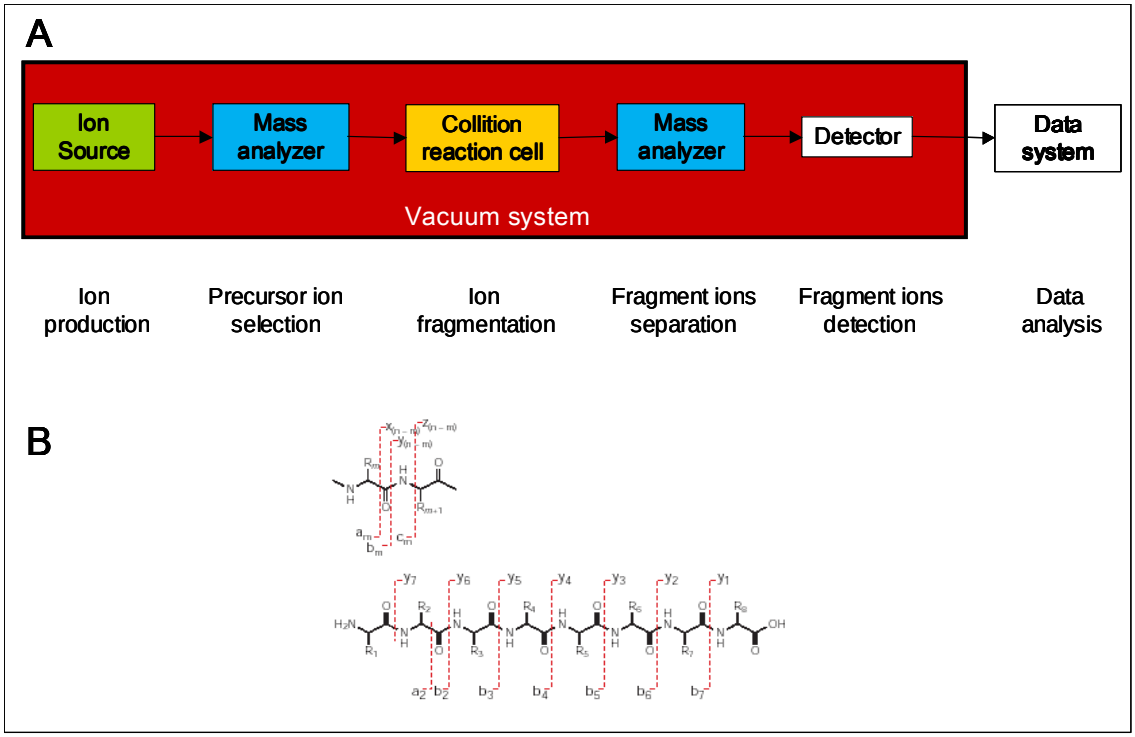

Figure 9. A: Steps for tandem mass analysis. **B**: Nomenclature of fragment ions produced during tandem mass spectrometry. Fragment ions are named according to the specific broken bond and to the side of the original peptide that keeps the charge; *a*, *b*, and *c* ions correspond to N-terminal fragments and *x*, *y*, and *z* ions correspond to C-terminal fragments of the peptide.

2.2. Choice of adequate samples

From a technical and methodological perspective, a pipeline for new biomarker discovery was proposed by N. Leigh Anderson [Anderson 2005b]. It required three stages: 1) discovery, 2) verification and validation, and 3) clinical implementation, where each stage includes different analytical techniques. In the discovery stage, proteomic techniques are usually used in order to directly analyze blood samples of diseased patients and controls. Proteomic analysis of plasma samples allowed discovering of potential biomarkers for cardiovascular diseases, such as acute coronary syndrome [Mateos-Caceres *et al.* 2004; Donahue *et al.* 2006; Peronnet *et al.* 2006] or peripheral arterial disease [Wilson *et al.* 2007].

However, to date, there are few, if any, new biomarkers that have been resulted in a commercial product by completing the proteomic process chain of identification, validation in clinical trials, and approval by regulatory agencies [Zolg 2006]. Recently, a further stage was proposed to be included into the biomarker discovery pipeline concerning studies only using plasma samples for biomarker discovery. In order to shorten the list of candidate biomarkers usually gained in these experiments, it should be mandatory to carefully select the tissue and/or cell type of interest from in the discovery phase and to focus on the proteins functionally involved in the pathological processes of the affected cells [Poschmann *et al.* 2009] (Figure 10). This new stage is based on the idea that candidate biomarkers are more abundant at their place of origin and they are released from diseased tissue into the blood stream [Meyer *et al.* 2007].

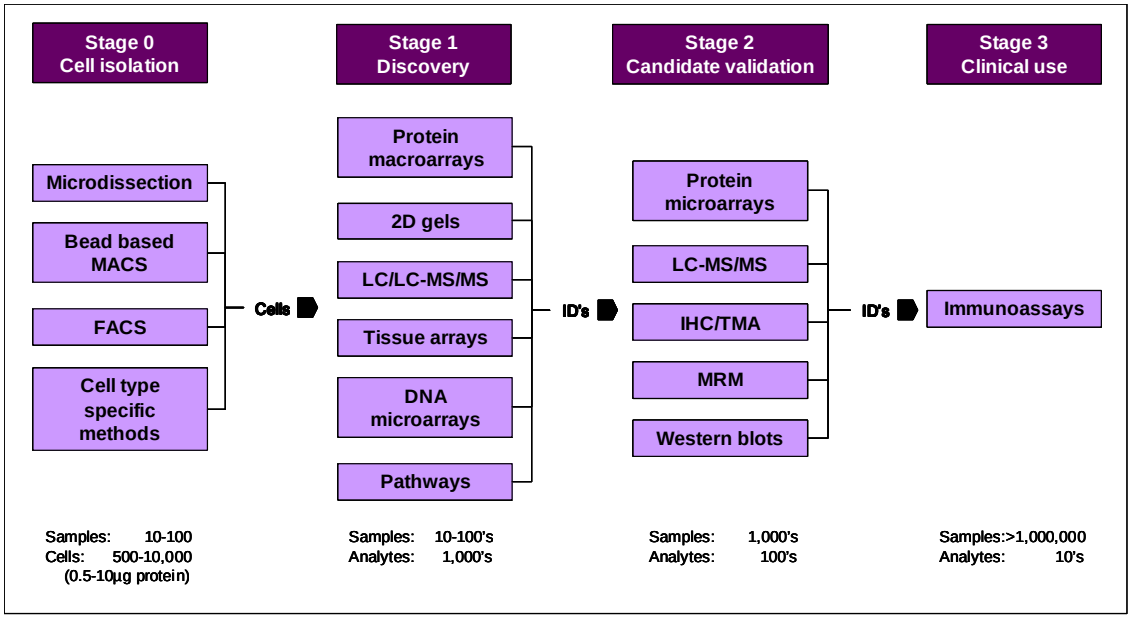

Figure 10. Schematic diagram of the four-stage diagnostic pipeline proposed by Anderson [Anderson 2005b] and later modified by Poschmann *et al*. [Poschmann *et al.* 2009]. Technologies, number of analytes to be potential biomarker candidates, and sample size are different in every stage.

In summary, candidate protein biomarkers should be first identified in specific cells from diseased tissue. These proteins, involved in the mechanisms of disease development are then selected and complementary analysis of plasma proteome leads to the first step of the potential biomarker validation in clinical trials. Up to this end, looks fairly clear that biological samples to be analyzed during cardiovascular biomarker discovery by proteomic methods are cells involved in the pathology and blood-derived samples.

2.2.1. Cell samples involved in aneurismal disease

Regarding AAA, three main cellular types have been described to be involved in pathogenesis of aneurismal development: endothelial cells, smooth muscle cells (SMCs) and monocytes/macrophages [Sho *et al.* 2005].

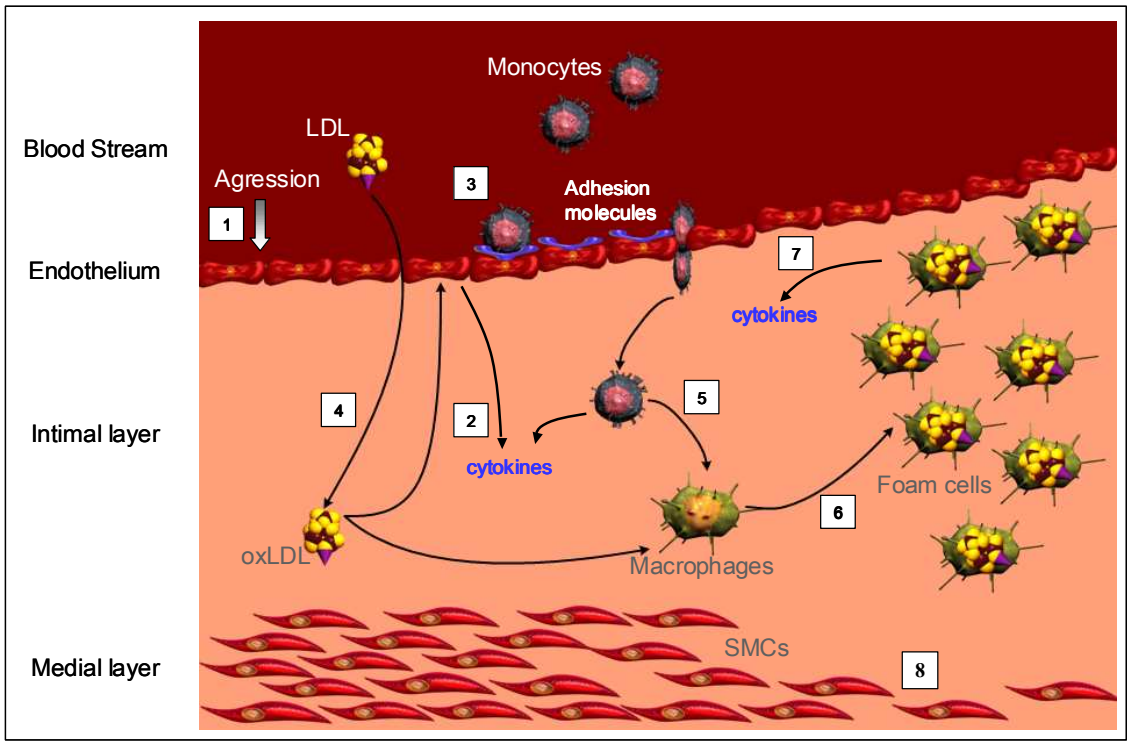

Figure 11. Atherosclerotic plaque formation in AAA.

A schematic representation of the atherosclerotic pathology associated to AAA development in the arterial wall is illustrated in Figure 11. When endothelium comes into contact with aggression molecules (1), endothelial cells undergo inflammatory activation and release cytokines (2) and inflammatory factors like adhesion molecules that promote the adhesion of blood leukocytes and monocytes to the inner surface of the arterial wall (3) [Libby 2002]. Endothelial cells are located at the border between the blood stream and the vessel wall, so they are responsible for most of the trafficking processes through the vessel wall. At the same time, (4) plasma low density lipoproteins (LDL) are transported across the intact endothelium and become trapped in the extracellular matrix where they become oxidized (oxLDL) [Witztum *et al.* 1991]. Once monocytes adhere to the endothelial layer, they penetrate into the aortic wall and there are activated into macrophages (5). OxLDL molecules are absorbed by macrophages and this process gives rise to the arterial foam cell formation (6). The foam cells secrete inflammatory cytokines (7) that amplify the local inflammatory response in an auto-feeding process since these cytokines will increase monocyte recruitment

into the aortic wall. Thus, endothelial cells, since they are forming the main barrier between blood and arterial wall, play a key role in trafficking of migratory inflammatory cells into the vessel wall. Macrophages are involved in inflammation and further release of MMPs (already discussed in section 1.3.) and smooth muscle cells are the main responsible for extracellular matrix regeneration. Further, SMC depletion from the media layer of the aneurismal aortic wall (8) contributes to AAA pathology [Thompson *et al.* 1997; Henderson *et al.* 1999].

So taking into account pathogenesis of arterial wall, expression levels, and PTMs of proteins in endothelial cells, vascular SMCs and macrophages from aneurismal tissue will probably be the phenotype of interest in biomarker discovery for AAA screening, diagnosis and prognosis. Cells infiltrated in the ILT, such as platelets ans neutrophils, may also be interesting to study since the thrombus is in permanent interaction with the blood stream, so released proteins could be easiest detected. Moreover, comparison between proteomes of this cell type in AAA patients vs. controls will also contribute to the better understanding of the physiopathology of AAA. Endothelial cells are too scarce in the aortic wall to be isolated and culture from an aortic biopsy. Our group has experience analyzing both human macrophages [Pinet *et al.* 2003; Dupont *et al.* 2004; Slomianny *et al.* 2006; Dupont *et al.* 2008] and arterial smooth muscle cells [Dupont *et al.* 2005] by gel-based proteomic techniques.

2.2.2. Body fluid samples

2.2.2.1. Plasma and serum

Classically, biological markers are required to be detectable in body fluids like urine or plasma and serum. Concerning AAA, since pathological mechanisms take place in the main blood vessel of the human body, it is logically to think that the main proteins involved in the pathology will be released directly into blood and will be easily detected. Two blood-derived samples are commonly used in clinics for biomarker analysis: serum and plasma. A plasma sample is obtained in the presence of an anticoagulant, usually EDTA, sodium citrate, or heparin, and centrifuged to remove blood cells. However, in the absence of an anticoagulant, a serum sample is obtained after blood is centrifuged and blood clots and cellular elements are removed [Schwarze *et al.* 2009]. It is necessary to standardize sample handling and preparation across the whole study and decide whether to use plasma or serum for proteomic analysis since protein composition in both samples is largely different [Issaq *et al.* 2007] and protocols to obtain plasma or serum can also differ from one lab to another. One could hypothesize that, since plasma requires a simpler procedure to be obtained, reproducibility among different laboratories could be achieved more easily than in the case of serum samples

in which the removal of blood clot would implicate removal of other proteins interacting specifically or not with the fibrin clot [Issaq *et al.* 2007]. However, the use of different anticoagulants generates different proteomic profiles [Banks *et al.* 2005]. Consequently, it is very important to utilize the same anticoagulant over time and over different laboratories involved in sample collection during the clinical study to obtain plasma samples that can be comparable.

Also, HUPO/Plasma Proteome Project Specimens Committee concluded that plasma was preferable to serum, due to less degradation *ex vivo* [Omenn *et al.* 2005; Tammen *et al.* 2005; Misek *et al.* 2005]. However, plasma proteome is the most complex human-derived proteome [Anderson *et al.* 2002] and also the most challenging to analyze. The main issue in plasma proteome analysis is the dynamic range of protein concentration that achieves 12 orders of magnitude [Anderson *et al.* 2002]. Indeed, 22 proteins make up to 99% of total protein amount in plasma [Anderson *et al.* 2004] (Figure 12). Thus, the challenging analysis of the remaining 1%, known as “deep proteome”, which includes thousands of different proteins [Righetti *et al.* 2006], involve a mandatory step of depletion of major proteins before sample analysis.

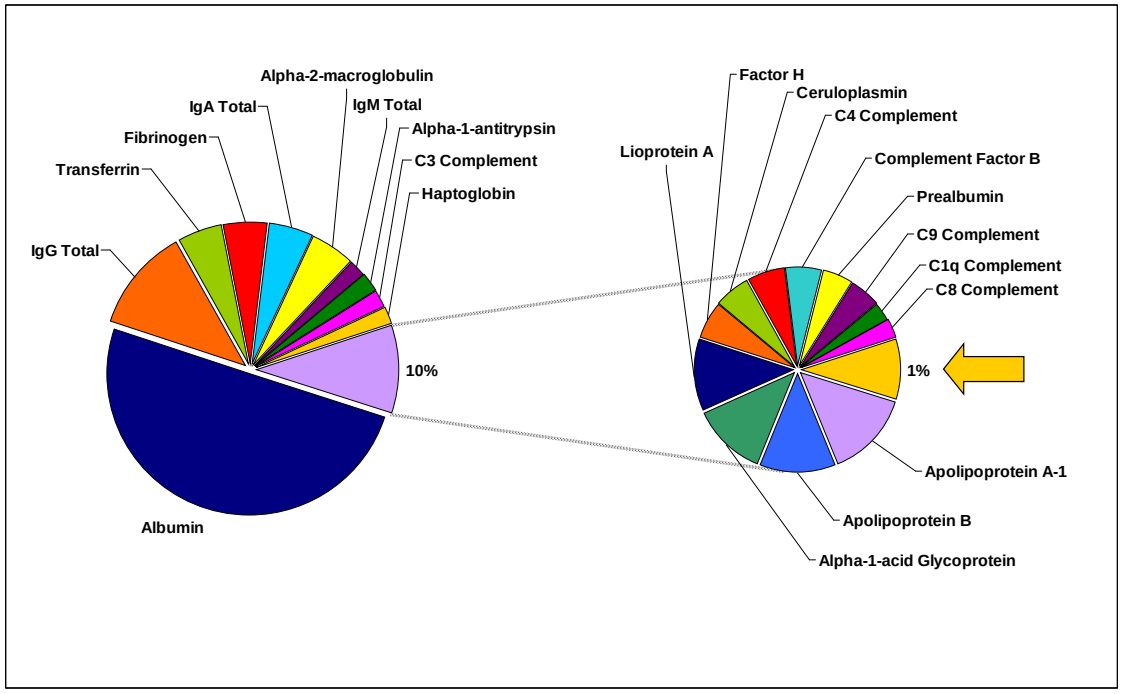

Figure 12. Dynamic range of protein concentration in human plasma/serum sample. Twenty two proteins make up to 99% of total protein amount while the deep proteome, consisting of the rest 1%, includes thousands of proteins. Adapted from [Issaq *et al.* 2007].

Thus, the depletion of abundant proteins allows for the reduction of dynamic range as well as detection and identification of low abundance proteins. Cardiovascular biomarkers are presumably among those low abundance proteins. Historically, immunodepletion has been used successfully in order to remove high-abundance proteins from plasma or serum, specifically albumin. However, immunodepletion of albumin involves simultaneous depletion of another 63 proteins bound to albumin [Zhou *et al.* 2004], including cytokines [Granger *et al.* 2005]. Recently, a new alternative approach, Protein Equalizer™ technology, was developed in order to compress the protein concentration range of plasma or serum, through the simultaneous one-step dilution of high-abundance proteins and concentration of low-abundance proteins [Guerrier *et al.* 2006]. This technology uses a large, highly diverse library of combinatorial hexapeptide ligands bound to a chromatographic support [Thulasiraman *et al.* 2005] and has been successfully used to study the deep proteome of serum from lung cancer patients [Au *et al.* 2007] and both plasma and serum samples from patients suffering left-ventricular remodeling (Fertin M, unpublished results). Once more the question of which depletion technique should be used in plasma proteome analysis is not yet answered but, by the moment, what one should keep in mind is that the technique chosen has to be applied the same way along the study and that results may be different if another technique is applied.

2.2.2.2. Urine

We could also consider urine which, compared to blood samples, has the advantage that it does not need the use of an invasive method to be obtained. Since protein content in urine come from filtration of blood, one could believe that proteins are similarly represented in both types of biological fluids and this can be noticed in most abundant proteins. For instance, albumin accounts for 55% of total plasma proteins content [Anderson *et al.* 2002] and is still the principal urinary protein measured [Barratt *et al.* 2007]. However, only 30% of urine proteins come from blood stream [Decramer *et al.* 2008a], the rest are proteins coming from the kidney and the urinary tract [Decramer *et al.* 2008a]. Thus, the use of urine is more useful for detection of biological markers in nephrology [Decramer *et al.* 2008b], renal disease [Dihazi *et al.* 2007] and prostate cancer [Downes *et al.* 2007] rather than cardiovascular diseases. Nevertheless, a recent study showed that coronary artery disease can be diagnosed with high confidence through the analysis of urinary proteome samples [Zimmerli *et al.* 2008]. Also, urine has a high amount of peptides and low molecular proteins that could be directly analyzed by MS without previous digestion [Decramer *et al.* 2008a]. Regarding to urinary peptidome, it seems that it may reflect the degree of turnover of the extracellular matrix. This hypothesis has been generated as a result of the observation that the most abundant urinary

peptides (based on ion counting) are not, as expected, the abundant proteins like albumin or uromodulin but specific collagen degradation products [Coon *et al.* 2008]. Consequently these peptides may be derived from ECM turnover. Thus, changes in this turnover also result in indicative changes in urinary peptides, which may serve as a very specific indicator [Decramer *et al.* 2008a]. So, considering that the main physiological effect of aneurismal disease is ECM degradation, why do not use the urinary peptidome to follow progression of AAA? This challenging question has not been yet addressed and plasma analysis is still the most widely strategy for AAA biomarker discovery.

In any case, it is clear that because of the complexity of human plasma it is necessary to conduct complementary analysis of cell samples coming from diseased tissue in order to achieve biomarker discovery.

3. Differential proteomic analysis

Differential proteomic analysis consists on the comparison of protein profiles from various samples obtained in different conditions in order to identify proteins differentially expressed without any *a priori* hypothesis [Cieniewski-Bernard *et al.* 2008]. There are many different workflows and methods that can be used to perform differential proteomic analysis but only a general overview of proteomic strategies used for biomarker discovery of AAA during the PhD work presented in this manuscript will be described.

3.1. Classical proteomics: 2D-PAGE followed by MALDI-TOF MS

Classically, differential proteomic analysis involves: 1) a separation step in which proteins of interest are separated by 2D-PAGE; 2) visualization of protein separated in the 2D-gel; 3) image analysis of the digitized 2D-gels; 4) in-gel digestion of differentially expressed proteins; and 5) identification of each protein by peptide mass fingerprint using MALDI-TOF mass spectrometry. Identification of proteins can be validated by performing MS/MS analysis that allows sequencing of protein peptides. Complementary methods like ELISA or western blot are usually used to validate protein expression found by proteomic analysis.

Two-dimensional-gels can vary on size, polyacrylamide/acrylamide ratio, and the range of pH used during isoelectric focusing. Depending on these parameters more or less resolution is achieved for protein separation. Also, the choice of gel characteristics is related to which are the specific proteins of interest in the sample. Interestingly, 2D-PAGE easily allows detection of PTMs, which cannot be predicted from genome sequences. Thus, thousands of proteins can be simultaneously separated and PTMs can be detected in a wide range of different samples:

from bacteria [Norais *et al.* 2007; Cole *et al.* 2008], yeast [Chery *et al.* 2001] and cells [Al Ghoul *et al.* 2008], to plasma [Darde *et al.* 2007; Cho *et al.* 2008; Chatterji *et al.* 2009] and plant extracts [Danchenko *et al.* 2009; Di Carli *et al.* 2009]. Despite the potential and resolution of 2-DE, it remains a labor-intensive technique that requires qualified personal to obtain reproducible results [Monteoliva *et al.* 2004]. In general, variations between gels are due to heterogeneities during acrylamide polymerization, electrical, pH, and thermal fluctuations across the gels during the run and variable precipitation of samples in isoelectric focusing process [Timms *et al.* 2008]. Furthermore, the study of very alkaline, hydrophobic and low or high Mr proteins is still a challenge that skips from gel-based proteomic analysis. However, there is no doubt that 2D-PAGE will remain an essential technique for characterization of proteomes for many years to come in spite of its weaknesses [Issaq *et al.* 2008].

3.1.1. Classical silver staining vs. chemical modification with fluorescent labeling

Proteins in analytical 2D-gels can be visualized by different methods and protocols. Silver staining [Heukeshoven *et al.* 1988] is the method classically used to detect polypeptidic spots since development of 2D-PAGE. Today, more than 100 different variants of silver staining protocols exist and they can be classified into two main categories depending on the reagent used for silver impregnation: silver nitrate and silver-ammonia protocols [Miller *et al.* 2006]. Both kinds of methodologies follow the same logic: proteins bind silver ions, which can be reduced under appropriate conditions to build up a visible image made of finely divided silver metal. Classical silver staining combines high sensitivity in the low nanogram range with very simple and cheap equipment and chemicals [Chevallet *et al.* 2006]. Later, to overcome reproducibility of classical silver stained 2D-gels, a fluorescent covalent labeling was developed and named DIGE from *difference gel electrophoresis* [Unlu *et al.* 1997]. The principle of DIGE consists of the co-migration in the same 2D-gel of different samples that have been labeled with different fluorescent cyanine dyes differing in their excitation and emission wavelengths. So methodological variations in spots positions and protein abundance are highly reduced, and, consequently, image analysis is facilitated considerably [Gorg *et al.* 2004]. The first DIGE dyes, called minimal dyes, are N-hydroxysuccinimidyl ester derivates of cyanine dyes Cy2, Cy3, and Cy5, developed to react with primary amino groups (N-terminal α-amino and lysine ε-amino groups) in the target protein sample. These dyes have a detection limit of 100-200 pg and a linear dynamic range of 3-5 orders of magnitude [Tonge *et al.* 2001]. Thus, linear dynamic range of silver staining detection, that is 2 orders of magnitude [Heukeshoven *et al.* 1988], was dramatically improved. However, in some studies where protein samples are scarce, performance of classical silver stained 2D-gel or labeling with minimal DIGE dyes is not

possible due to the required amount of protein, 100 and 40 µg respectively. This problem has been solved by the development of fluorescent saturation DIGE dyes that allow 2D-PAGE with only 5 µg of protein [Shaw *et al.* 2003]. In this case, maleimide derivates of cyanine dyes Cy3 and Cy5 are able to react with reduced thiol groups of cysteine residues in the target protein sample (Figure 13). Saturation DIGE labeling has a similar linear dynamic range that minimal DIGE labeling while increasing sensitivity of detection to 5-10 pg of protein. Both minimal and saturation DIGE labeling required specific equipment for image acquisition.

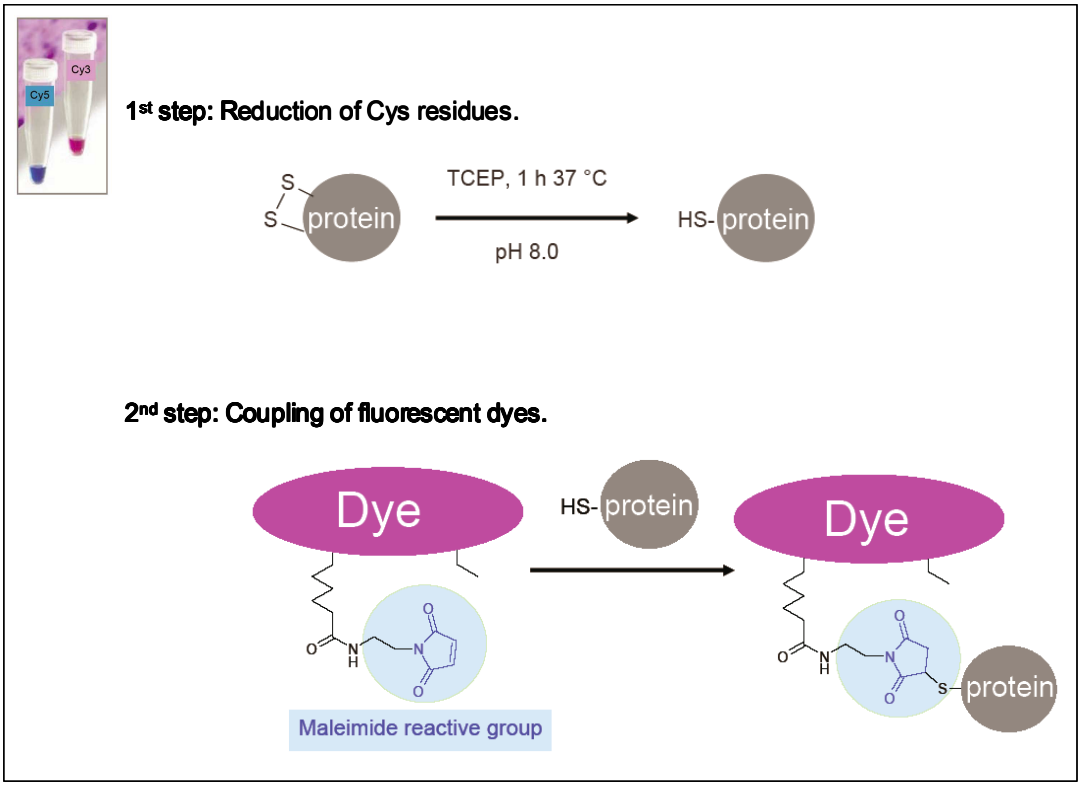

Figure 13. Labeling steps for protein derivatization with saturation DIGE dyes (Adapted from GE Healthcare).

Furthermore, from a point of view of rare sample analysis, the introduction of saturation DIGE labeling technique to differential proteomic analysis has been revolutionary and has allowed proteomic study of diverse scarce samples obtained from microdissected tissue [Kondo *et al.* 2006; Pennington *et al.* 2008; Sitek *et al.* 2008]. Thus, protein labeling with saturation DIGE dyes for further analysis of 2D-gels is a key methodology used during protein biomarker discovery of human diseases.

3.1.2. Image analysis

Image analysis by specifically designed software is one of the most important and time consuming steps during 2D-gel based proteomic analysis. Currently, several 2D-image analysis

software packages are commercially available. The traditional workflow for a 2D-gel software package is 1) preprocessing of the gel images in each group, i.e. normalization, cropping and background subtraction; 2) spot detection and expression quantification; 3) alignment and matching of spots within the gels to a reference image, and 4) interpretation of differentially expressed spots through statistical analysis. This workflow implies a high subjective contribution of the user to each step of the analysis, being gel alignment after spot detection the most critical step. However, a new software generation has been developed to reduce user intervention during 2D-gel image analysis, and a new workflow has been designed: 1) after a reference image is chosen, the other 2D-gel images are aligned and spots are matched; 2) spot volumes are calculated and normalized in each image; 3) gels are selected for each group; and 4) differences in protein spots are evaluated by statistics. So the main differences between both types of software are the order in which spot detection and image alignment are performed.

One example of the new software generation is Progenesis SameSpots software (Nonlinear Dynamics, Newcastle upon Tyne, UK). This is an user-friendly software that follows the workflow described above in which alignment of spots is performed before spot detection. The order of these steps makes that only spots detected in a minimum number (established by software algorithms) of aligned gels are considered for the differential expression analysis. Thus manual verification and clean-up of the gels are less time consuming steps during bioinformatic analysis.

Despite continuous improvements in algorithms for spot matching, quantification, analysis, and integration of data meaning, software for bioinformatic analysis of 2D-gel images are still a long way from totally automatic systems that do not require user intervention [Gorg *et al.* 2004]. Objectivity and a critical regard are important for the correct processing and interpretation of 2D-gel images.

3.1.3. MALDI-TOF MS

Mass spectrometers that combined matrix-assisted laser desorption/ionization (MALDI) [Karas *et al.* 1987] with time of flight (TOF) mass analyzer are usually used to identify in-gel digested proteins analyzed by 2D-gel based methodologies.

In ionization by MALDI, the sample analytes are firstly co-crystallize with molecules of matrix on a MALDI plate. Then, a laser beam (normally a nitrogen laser with a wavelength of 337 nm) fires at the crystals and matrix molecules trap the energy of the laser, and ionize analyte molecules. There are several types of matrix but the most commonly used for

proteomic analysis of digested proteins is α-cyano-4-hydroxycinnamic acid. Matrix also protects analyte molecules from thermal decomposition. Ionization by MALDI normally generates single charged ions.

After ionization, peptides go into the mass analyzer to be separated according to their m/z values. Indeed, in TOF mass analyzers, generated ions are accelerated by a known electric field. The velocity of the ions depends on the m/z ratio so ions with different values of m/z reach the detector at different times. Consequently, single charged peptides with low molecular weights travel faster than single charged peptides with high molecular weights. In order to increase resolution between ions with the same m/z values but different kinetic energies, a reflectron was introduced in TOF mass analyzers.

Spectra obtained by MALDI-TOF mass spectrometry are usually used to identify proteins by peptide mass fingerprinting (PMF). Excised spots coming from 2D-gels can contain several proteins, and this protein mixture is usually enriched in only one or two proteins. PMF is based on the assumption that peptides resulting from digestion of a gel spot belong to the most abundant proteins in that spot. Thus, m/z values of peptides corresponding to the most abundant protein are represented in the spectra. Most of the time, trypsin is used as proteolytic enzyme for protein digestion before MS analysis. Thus, cleavage sites are known and experimental m/z values of digested peptides can be compared to m/z values of theoretical digestions of protein sequences in a data base.

3.2. LC-MS/MS methods

Mass spectrometers that use electrospray ionization (ESI) [Fenn *et al.* 1989] are usually used to analyze complex mixtures of peptides because its capability to be coupled to liquid-based separation tools like chromatographic separation systems (LC). In ESI ionization analytes are dissolved in a liquid phase that goes through a capillary tube. A strong electric field is applied at the end of the capillary tube and sample solution is dispersed into a fine aerosol made of charged droplets. These droplets go under vacuum, charge increases on the droplets, and ions enter the mass analyzer. ESI ionization generates multiple charged ions so their m/z ratio is decreased compared to singly charged analytes.

Many types of mass analyzers can be coupled with ESI ionizers. However, ion-trap analyzers are the most common to be combined with ESI. In ion-traps, ions are captured under the action of an electric or magnetic field and scanning through a range of voltage allows ion release, according to their m/z values, for further detection. A disadvantage of three-dimensional ion-traps is their relatively low mass accuracy that has been overcome by the

'linear' or 'two-dimensional' ion-traps [Hager 2003; Schwartz *et al.* 2002], in which ions are stored in a cylindrical volume that is considerably larger than that of the traditional, three-dimensional ion traps, allowing increased sensitivity, resolution and mass accuracy [Aebersold *et al.* 2003]. Different designs of MS instruments allow MS as well as MS/MS analysis. Thus, methods which consist of a first chromatographic separation of peptides coupled to mass spectrometry to perform MS/MS analysis are popularly called LC-MS/MS methods (Figure 14). In order to increase dynamic range of detection, peptide samples are usually fractionated previous MS analysis.

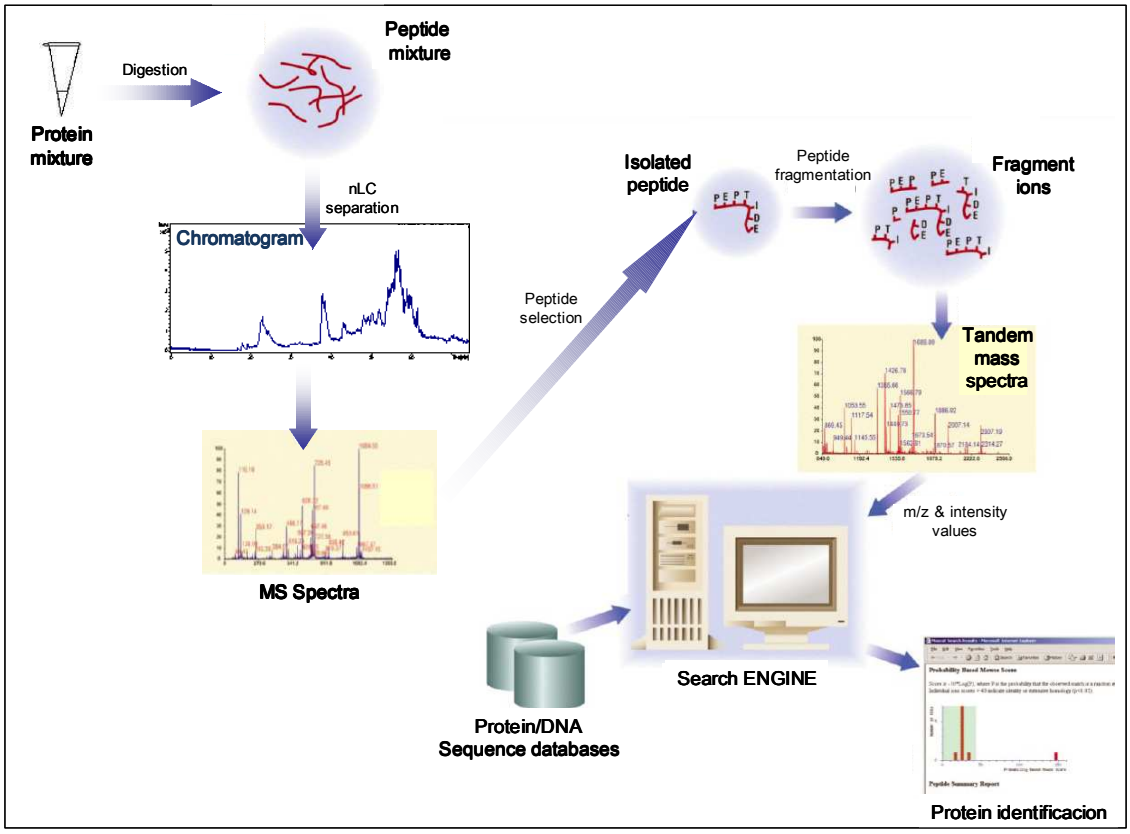

Figure 14. Typical workflow for protein identification when applying LC-MS/MS proteomic analysis. Protein mixture is digested in solution. Then, the obtained peptide mixture is separated by liquid chromatography and analyzed by tandem mass spectrometry. MS spectra of all peptides contained in a chromatographic peak at a certain time is obtained. After that, one peptide is isolated and fragmented, and m/z values of fragment ions are measured to generate a tandem mass spectra. m/z and intensity values of the fragments are compared against sequence databases corresponding to the fragmented peptide (Adapted from MATRIX SCIENCE).

Mass spectrometry has suffered drastic improvements during last years and many LC-MS/MS methodologies have been developed for both identification and quantification of peptides and proteins.

3.2.1. Data-independent acquisition

Most of the time, precursor ion selection and isolation for further tandem mass analysis is performed in a data-dependent acquisition mode, in which only the most abundant ionized species from each MS survey scan are selected. In order to prevent reselection of the same most intense ion over and over again, it is necessary to apply a complementary method referred to as "dynamic exclusion" [Gatlin *et al.* 2000]. Thus, acquisition parameters are established and determine how many times at the most an ion can be selected during a certain period of time. Combination of both methods improves coverage and dynamic range during proteome analysis. However, numerous studies show that full coverage of peptides in a complex mixture is far to be achieved, considering the non-reproducible nature of peptides detected in replicate analysis [Liu *et al.* 2004].

Recently, a new method referred to as "precursor acquisition independent from ion count" (PAcIFIC) [Panchaud *et al.* 2009] has been successfully applied to overcome the dynamic range and peptide coverage problems generated due to data-dependent acquisitions of MS data. PAcIFIC methodology consists on the acquisition of tandem mass spectra at every m/z value (i.e. m/z "channel") without regard for whether a precursor ion is observed or not. Actually, no precursor ion scans are even observed. The application of PAcIFIC for proteomic analysis of a sample replicate involves several injections of the peptide sample. During the first injection, the ion trap is used to perform data-independent collision induced dissociation (CID) at each of 10 continuous 1.5 m/z intervals across a range of 15 m/z per LC-MS analysis using a 2.5 m/z isolation width. In the next analysis, a new 15 m/z range is examined in an identical fashion and so on until the considered precursor ion m/z range of 400-1400 units is covered. Summarizing, all peptides that have a m/z value included in the precursor ion range of 400-1400 units are at some point selected for tandem mass analysis. This method showed to provide superior protein sequence and proteome coverage compared to data-dependent acquisition methods in both cell samples and plasma.

3.2.2. MS-based quantitative methods

As already described in section 2.1.4., MS-based quantitative methods can be classified into two groups: isotopic labeling methods and label-free methods. One example of each type is described below.

3.2.2.1. Spectral count

The label-free quantification approaches are based on the correlation of either the mass spectrometric signal of intact proteolytic peptides or the number of peptide sequencing

events with the relative or absolute protein quantity [Bantscheff *et al.* 2007]. Regarding data treatment, one of the easiest methods to be applied is spectral count analysis. Quantification by spectral count [Liu *et al.* 2004] is based on the empirical observation that the number of tandem MS spectra collected for peptides of a particular protein is correlated to the abundance of that protein in the sample. This quantitative method has been applied to perform relative quantification of proteomes from different types of samples like yeast [Liu *et al.* 2004], smooth muscle cells [Gao *et al.* 2008] or plant extracts [Stevenson *et al.* 2009], whose MS analysis has been done by applying data-dependent acquisition mode. Thus, number of spectral counts found for a certain protein may be affected by the acquisition parameters to select precursor ions, for instance dynamic exclusion parameters. Indeed, a recent study reports the effect of dynamic exclusion duration on spectral count based quantitative proteomics [Zhang *et al.* 2009]. It seems that different durations during dynamic exclusion leads to different numbers of spectra assigned to a certain protein. Consequently, one could think that this type of quantitative approaches is more appropriate when peptide samples have been analyzed by MS methods like PAcIFIC in which dynamic exclusion is not required and MS spectra are obtained independently of the intensity profile of precursor ions. However, the statistical framework for treatment of quantitative data obtained by spectral count analysis is still evolving and needs to be better established [Zhang *et al.* 2006; Choi *et al.* 2008; Carvalho *et al.* 2008; Zhang *et al.* 2009].

3.2.2.2. Isotopic labeling with isobaric tags

Isobaric tags are a multiplexed set of reagents that have the same chemical structure and molecular weight but a different isotopic composition and distribution. That makes that the same derivatized peptides from different samples, which have been labeled with different tags, are also isobaric and chromatographically indistinguishable. However, fragmentation of labeled peptides during collision induced dissociation (CID) for MS/MS analysis gives rise to the generation of reporter ions. The ratio between intensities of reporter ions can be used to quantify peptides over the different proteomic samples (Figure 15B).

This type of technique was developed for the first time by Applied Biosystems and was referred to as “isobaric tag for relative and absolute quantitation” (iTRAQ) [Ross *et al.* 2004]. Later, similar reagents were also commercially available, like the “tandem mass tags” (TMT) [Dayon *et al.* 2008] that are sold by Thermo Scientific and whose application have been shown successful results [Viner *et al.* 2009; van Ulsen *et al.* 2009]. The TMTduplex label reagent set is composed of two isobaric tags that generate reporter ions at different m/z values, i.e. 126 and 127 (Figure 15A).

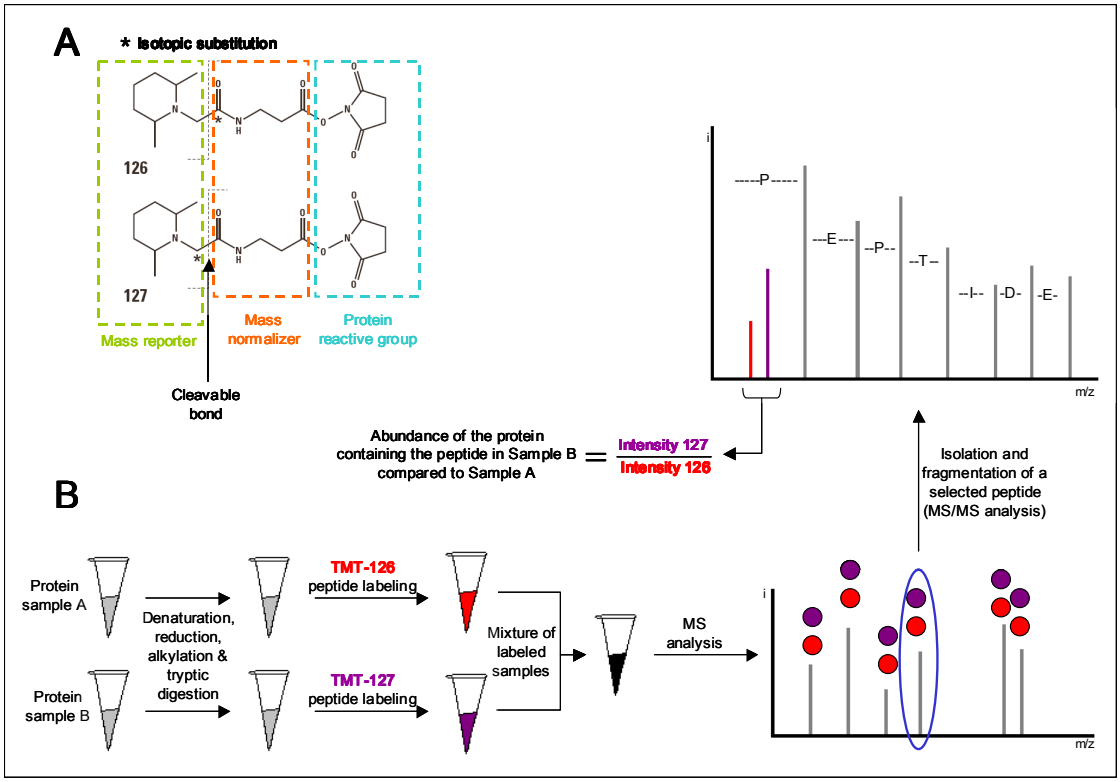

Figure 15. A: Chemical structure of TMTduplex reagents. Each isobaric reagent is composed of an amine-reactive N-hydroxysuccinimidyl ester group, a spacer chain that is used to compensate the mass differences due to the isotopic substitutions, and a MS/MS reporter that allows identification and quantification of proteins in each sample. **B**: Workflow for TMT labeling and analysis. First sample proteins are denaturized, Cys residues are usually reduced and alkylated, and trypsin digestion is performed. Then, peptide digests are labeled with the different TMT reagents and peptide mixtures of both samples are combined to be analyzed by LC-MS/MS. In MS spectra the same peptides that come from different labeled samples have the same m/z value. Later, MS/MS analysis of a selected peptide allows sequencing for further identification, and reveals reporter ions at the low mass region of the spectra, which intensities are used to perform relative quantification.

RESULTS

IV. RESULTS

1. Objectives

In this work we have used differential proteomic techniques in order to analyze and compare human blood samples (plasma) and cells (smooth muscle cells and monocyte-differentiated macrophages) from patients presenting an abdominal aortic aneurysm (AAA) to unaffected patients. These analyses pursue toward the following objectives:

a) To identify and evaluate potential biological markers for AAA screening, that will ensure early diagnosis and subsequent early treatment of this fatal disease.

b) To provide a better understanding of the physiopathological mechanisms involved in the evolution of AAA through the interpretation of the results in differential proteomic analysis

2. Clinical population: CORONA

CORONA clinical protocol (CCPPRB n°CP 01/96 of 04/12/01) has included 265 patients with advanced coronary disease undergoing coronary bypass grafting from 2002 to 2006 in CHRU of Lille (France). Vascular and abdominal echography examination was performed after surgery to check the quality of bypass and to measure the aorta diameter and check for presence of AAA. At the time of recruitment, AAA was detected in 17 patients, representing 6.4% of the population with a mean age of 64.6 years. Blood samples were obtained from all patients before surgery undergoing. Blood sample processing allowed obtaining of plasma and serum as well as isolation of monocytes that were *in vitro* differentiated into macrophages as described by Boyum *et al*. [Boyum 1968]. Also for each patient, smooth muscle cells were isolated from a residual segment of internal mammary artery used for the bypass, and cultured *in vitro*.

2.1. Previous analysis of the CORONA clinical population

All enrolled patients were prospectively interviewed before undergoing bypass grafting upon admission at the hospital. All data were recorded and investigated in order to establish risk factors associated with the presence of AAA (Dupont A, personal communication). Thus, recorded data included usual anthropometric and cardiovascular risk factors: body-mass index, age, gender, smoking status, diabetes, arterial hypertension, hypercholesterolemia, hypertriglyceridemia and family history of cardiovascular diseases. Clinical data were also recorded, including history of myocardial infarction, stroke, symptomatic angina, peripheral arterial disease, AAA surgery and coronary angioplasty. Left ventricular ejection fraction, severity of coronary artery disease, other chronic illness and medication were also recorded. Moreover, carotid artery stenosis was evaluated

before surgery undergoing, and echography examination after surgery allowed measurement of the diameter of aorta and its major arterial branches. In this study, AAA was considered when aortic diameter was ≥30 mm or infra- to supra-renal ratio was >1.5. All other arterial aneurysms were considered when diameter was at least 1.5 times the diameter of the proximal adjacent segment. Table 1 presents a summary of the statistical analysis on the recorded data. From 265 patients, 48 had some unavailable information and were not used for the analysis of possible risk factors for the presence of AAA.

Table 1. Association between characteristics of patients with severe coronary artery disease undergoing coronary artery bypass grafting surgery and abdominal aortic aneurysm presence

Characteristics	**All patients n= 217**	**Non-AAA n= 202**	**AAA n= 15**	***p-Value* between AAA and no AAA**
Age, years	64 (56-73)	64 (56-73)	67 (58-75)	0.24
Gender, % female	28 (13)	28 (14)	0 (0)	0.12
BMI, kg/m^2	27 (25-30)	27 (25-30)	27 (24-30)	0.52
Diabetes mellitus, %	63 (29)	57 (28)	6 (40)	0.38
Arterial hypertension, %	143 (66)	134 (66)	9 (60)	0.62
Hypercholesterolemia, %	138 (64)	129 (64)	9 (60)	0.76
Hypertriglyceridemia, %	37 (17)	34 (17)	3 (20)	0.73
Smoking, current or past, %	**139 (64)**	**124 (61)**	**15 (100)**	**0.003**
Positive family history for CVD, %	81 (37)	73 (36)	8 (53)	0.18
LVEF < 50%, %	60 (28)	56 (30)	4 (33)	1.0
Unstable angina pectoris, %	36 (17)	35 (17)	1 (7)	0.35
Stable angina pectoris, %	82 (38)	79 (39)	3 (20)	0.14
Peripheral arterial disease, %	**28 (13)**	**22 (11)**	**6 (40)**	**0.006**
Stroke, %	19 (9)	17 (8)	2 (13)	0.63
Previous myocardial infarction, %	81 (37)	76 (38)	5 (33)	0.74
Previous coronary angioplasty, %	53 (24)	49 (24)	4 (27)	1.0
Carotid artery stenosis, %	**18 (8)**	**14 (7)**	**4 (27)**	**0.0075**
1-coronary vessel disease	26 (12)	25 (12.5)	1 (7)	1.0
2-coronary vessel disease	48 (22)	45 (22)	3 (20)	1.0
3 or 4-coronary vessel disease	143 (66)	132 (65.5)	11 (73)	0.52

Leukocytes, G/l	6.9 (5.9-8.1)	6.9 (5.9-8.1)	7.6 (5.7-9.0)	0.63
Fibrinogen, (g/l)	3.35 (2.9-4.0)	3.32 (2.9-4.0)	3.6 (3.2-4.1)	0.2
CRP, mg/l (n=213)	**1.75 (0.9-3.5)**	**1.69 (0.81-3.49)**	**2.75 (1.71-3.74)**	**0.053**
Aspirin	179 (82)	166 (82)	13 (87)	0.48
Beta-blocker therapy	158 (73)	148 (73)	10 (67)	0.56
Angiotensin converting enzyme inhibitor	101 (46)	95 (47)	6 (40)	0.79
Calcium antagonist treatment	54 (25)	51 (25)	3 (20)	0.77
Statin	169 (78)	157 (78)	12 (80)	1.0
Suprarenal aortic artery diameter (mm)	**18.2 ± 2.7**	**18.1 ± 2.6**	**20.6 ± 4.0**	**0.038**
Infrarenal aortic artery diameter (mm)	**18.4 ± 5.4**	**17.3 ± 2.6**	**35.3 ± 9.1**	**-**
Infra/suprarenal aortic diameters ratio	**0.99 ± 0.20**	**0.96 ± 0.11**	**1.64 ± 0.42**	**-**
Femoral artery diameter (mm)	**8.9 ± 1.5**	**8.8 ± 1.4**	**10.4 ± 1.9**	**0.008**
Popliteal artery diameter (mm)	**7.2 ± 1.5**	**7.1 ± 1.5**	**8.7 ± 1.5**	**0.0012**

Data are expressed as either median value (25^{th} to 75^{th} percentile range) or n (%). For external arterial diameters and infra/suprarenal ratio values are expressed as mean ± SD. Sample size is 217 unless otherwise indicated. AAA: abdominal aortic aneurysm, BMI: body mass index, CVD: cardiovascular diseases, CRP: C-reactive protein, LVEF: left ventricular ejection fraction.

To briefly conclude, significant statistical differences between data of aneurismal and non-aneurismal patients (in bold highlighted) indicated that patients with severe coronary artery disease, aged <75 years, with smoking history, and concomitant extracoronay atherosclerotic lesions present a particular high risk for AAA.

2.2. Description of the subpopulation used for the proteomic analysis

From the whole population included in CORONA clinical protocol, a subgroup of patients was selected to perform the proteomic analysis with their corresponding samples.

2.2.1. Patients matching for the proteomic analysis

Every one of the 17 AAA patients was paired with 3 non-AAA patients, according to their age, gender, tobacco consumption, arterial hypertension, diabetes and dyslipidemia. From these three non-AAA patients, one of them is perfectly coupled according to these characteristics, but the two others, while having the same characteristics, have slightly different biological and clinical values relative to the AAA patient.

2.2.2. Statistical analysis

According to the previous classification, the biological and clinical data of the CORONA sample set, composed of 68 plasma samples from 17 AAA and 51 non-AAA bypass patients, were analyzed. Since one of the data set size can be consider as small (<30), exact nonparametric inference tests were applied using StatXact.8 software. For continuous data, permutation test was used. For categorical data, Fisher's exact test was used to analyze binomial data and Pearson's Chi-square test was used for the analysis of multinomial data, i.e. smoking status, coronary vessel disease or angina pectoris. A summary of the statistical results on the evaluated data are presented in table 2.

Table 2. Baseline characteristics of the study population for the proteomic analysis

Characteristics	**Non-AAA** **n= 51**	**AAA** **n= 17**	***p-Value***
Biological data			
Age (years), mean value ± SD	63.52 ± 9.0	64.89 ± 9.9	0.59
Gender, n (% female)	0 (0)	0 (0)	ND
Anthropometric data, mean value ± SD			
Body mass index (kg/m^2)	26.60 ± 3.4	27.68 ± 4.2	0.28
Cardiovascular risk factors, n (%)			
Type 2 diabetes	15 (30)	5 (27.8)	0.59
Arterial hypertension	30 (60)	11 (61.1)	1
Dyslipidemia	34 (68)	11 (61.1)	0.69
Current smoking	6 (12)	4 (22.2)	0.32
Past smoking	35 (70)	13 (72.2)	
Positive family history for CVD	16 (32)	8 (44.4)	0.61
Personal history of CAD, n (%)			
Unstable angina pectoris	12 (24)	4 (22.2)	0.99
Stable angina pectoris	20 (40)	6 (33.3)	
Peripheral arterial disease	7 (14)	5 (27.8)	0.27
Stroke	3 (6)	2 (11.1)	1
Previous myocardial infarction	17 (34)	4 (22.2)	0.54
Carotid artery stenosis	13 (26)	5 (27.7)	1
1-coronary vessel disease	4 (8)	3 (16.7)	0.14

2-coronary vessel disease	12 (24)	8 (44.4)	
3-coronary vessel disease	33 (66)	7 (38.9)	
Clinical medication at time of inclusion, n (%)			
Aspirin	44 (88)	14 (77.8)	0.44
Beta-blocker therapy	38 (76)	12 (66.7)	0.53
Angiotensin converting enzyme inhibitor	22 (44)	8 (44.4)	1
Calcium antagonist treatment	14 (28)	4 (22.2)	0.76
Statin	42 (84)	12 (66.7)	0.17
Presence of other arterial aneurisms, n (%)			
Iliac aneurismal extension	0 (0)	4 (22.2)	0.0037
Femoral arterial aneurism	0 (0)	1 (5.5)	0.31
Popliteal arterial aneurism	0 (0)	0 (0)	ND

Data are expressed as either mean value ± Standard Deviation (SD) or number of patients (%). AAA: abdominal aortic aneurysm, CAD: coronary artery disease, CVD: cardiovascular diseases, ND: no difference.

For almost all evaluated data, statistical analysis showed that there are not significant differences between patients belonging to non-AAA group and patients belonging to AAA group. Only the number of patients presenting an aneurismal extension in the iliac arteries was significantly different ($p = 0.0037$) between both groups of patients, and this is not surprising considering that iliac arteries are formed at the terminus or the aorta in the abdominal region. Thus, it seems that the dilation process in patients with AAA were not exclusive for the aortic wall but for a larger location over the wall of the arterial system.

Taken together, similarities between biological and anthropometric data, cardiovascular risk factors, and personal history of CAD in both groups of patients suggest that the potential biomarkers found in the proteomic analysis will be more specific for AAA than in the case that control samples were from completely healthy patients since the presence of AAA is the main fact that differs from one group to the other.

3. Analysis of cell samples

Two types of human cell samples were obtained from patients recruited in the CORONA clinical protocol: blood monocytes that were *in vitro* differentiated into macrophages, and smooth muscle cells isolated from a residual segment of internal mammary artery used for the bypass, and cultured *in vitro*. Although these cell types represent the main cell types involved in AAA

development, it is important to notice that the origin differs. Ideally, the best choice would be the isolation of the cells from aneurysmal tissue. Unfortunately, patients included in CORONA clinical protocol did not undergo AAA repair, so aneurysmal biopsies were not available for cell isolation and further analysis. However, the study of monocyte-derived macrophages and SMCs from these patients was a great opportunity to characterize and compare proteomes between AAA patients and controls. Culture of obtained cells may have the limitation of changing cell phenotype and further protein expression. Despite this fact, similar studies in our group have shown that disease phenotype is still reflected after cell culture, at least for macrophages. Proteomic analysis of both cell types was performed by 2D-DIGE analysis with saturation labeling.

3.1. Macrophage proteomic analysis

3.1.1. Introduction

Macrophages are implicated in complicate inflammatory processes involved in AAA formation and development. Thus, in aneurismal pathology inflammatory macrophages which infiltrate the tunica media play an important role in release of MMPs and further degradation of the ECM, which is the main cause of the enlargement of the aortic diameter. Previous studies in our group have shown (LILAS study, not published) that disease phenotypes of circulating monocytes are maintained during *in vitro* differentiation into macrophages. According to this statement, protein extracts from monocytes-derived macrophages coming from patients recruited in CORONA clinical protocol were analyzed by 2D-DIGE in order to compare proteomic profiles between AAA and non-AAA samples.

3.1.2. 2D-DIGE analysis of human macrophage protein samples

Surprisingly, bioinformatic analysis of 2D-DIGE gels of macrophage samples revealed a technical bias during sample processing at the protein extraction step. This bias gave rise to a clearly modified proteomic profile in some of the macrophage samples, independent of the disease status classification (AAA and non-AAA) of the patients. These interesting results were recently published and are shown below. Next, 2D-DIGE gels of AAA and non-AAA samples were compared within each group of proteomic profiles according to the classification generated by the technical bias.

3.1.2.1. Manuscript: Impact of incomplete Dnase I treatment on human macrophage proteome analysis

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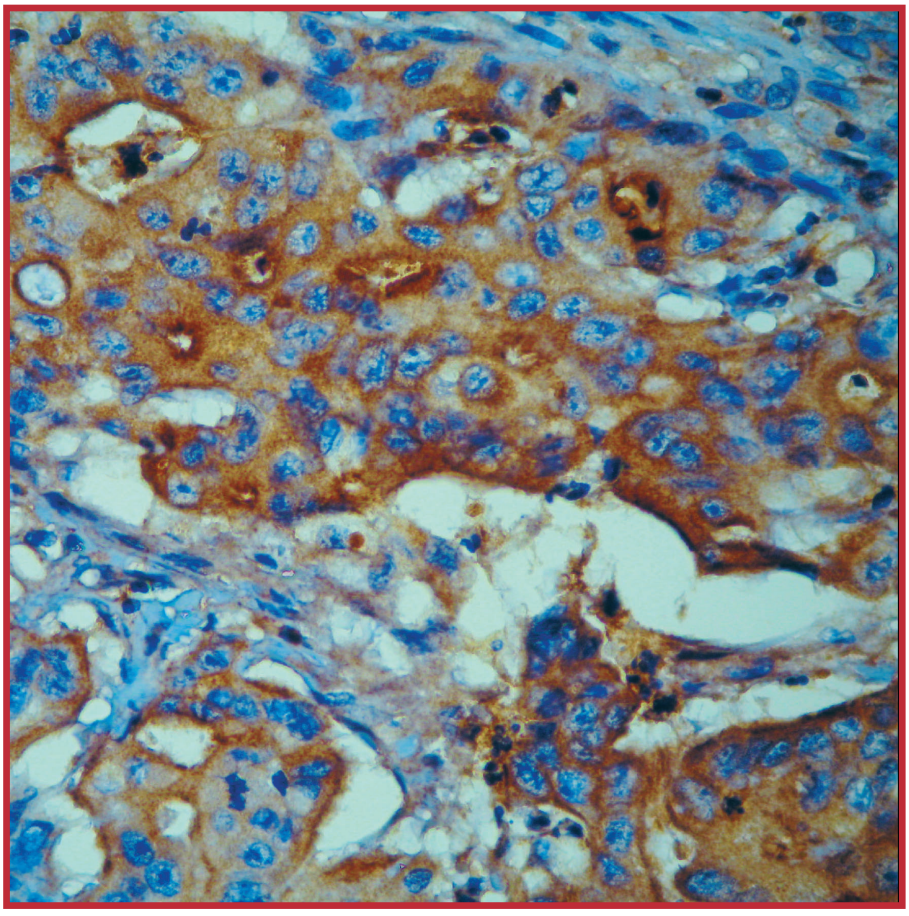

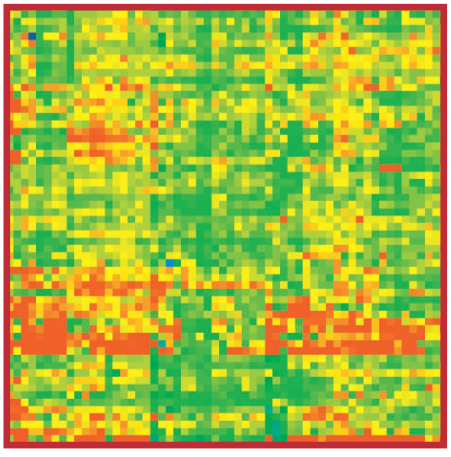

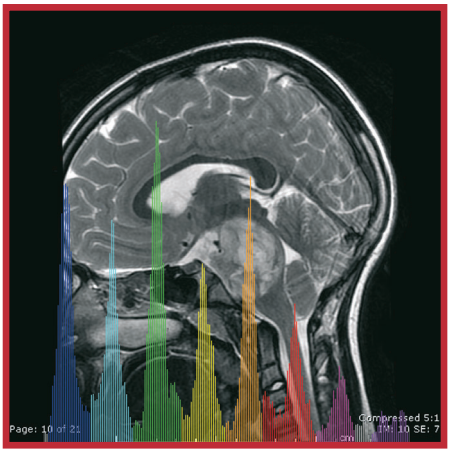

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RESEARCH ARTICLE

Impact of incomplete DNase I treatment on human macrophage proteome analysis

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The aim of our study was to analyze the proteomic pattern of human macrophages obtained over a 4 year period from blood donors. The purpose was to simulate a long-term clinical study to assess the application of 2-D DIGE technique for differential proteomic analysis of these scarce samples. Bioinformatic analysis of 2-D DIGE gels of 19 different cultures of macrophages assessed whether they did or did not contain at least specific five spots identified by MS as being or containing bovine deoxyribonuclease I (DNase I). Bovine DNase I was used during sample treatment to remove nucleic acids from protein extracts. Macrophages were classified in two groups, which appeared to be differentiated by the completeness of DNase I treatment. Further detailed analysis revealed a different proteomic pattern of macrophage protein samples according to the completeness of this treatment. The major group of proteins affected, accounting for one third of the differentially expressed proteins, included proteins involved in cell motion and actin cytoskeleton reorganization. The use of DNase I for the removal of nucleic acids from protein samples must be avoided in proteomic studies since it can generate bias in the analysis of protein expression patterns.

Keywords:
Deoxyribonuclease I / 2-D DIGE / Macrophage / MALDI-TOF / Proteome

1 Introduction

2-DE, combined with MS for protein identification, has become the proteomic technique most common applied in the study of protein expression pattern in diverse biological processes. It has also been applied to the analysis of a wide range of different samples, from bacteria [1–2], yeast [3] and cells [4], to plasma [5–7] and plant extracts [8–9]. In some studies where protein samples are scarce, classical 2-DE cannot be applied because it is insufficiently sensitive. This problem has been solved by the development of fluorescent saturation DIGE dyes [10–11] that allow 2-D gels that use only 5 μg of protein. Recently, 2-D saturation DIGE technology has also been used to analyze microdissected tissue [12–14]. Thus, thousands of proteins can now be simultaneously separated, and specific staining can be used to detect post-translational modifications [15]. High resolution of polypeptide spots requires not only good electrophoresis conditions, but also appropriate sample preparation. The latter generally depends on the nature of the sample.

Previous reports by our team described the application of silver-stained 2-DE to analyze the protein expression patterns of macrophages from blood samples [16]. Short-term studies and 2-DE allowed us to construct 2-D maps and databases of human macrophages characterized according to previously established morphological and biochemical criteria [17]. More recently, we used the 2-D DIGE technique to characterize proteins modulated by oxidized low-density

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Abbreviations: ABP, actin binding protein; **Cy,** cyanine; **DNase,** deoxyribonuclease; **GO,** gene ontology; **MDM,** monocyte-derived macrophage; **RNase,** ribonuclease

lipoprotein treatment of human macrophages [18]. 2-DE combined with saturation DIGE-labeling allows reproducible differential proteomic analysis of scarce protein samples and modulations of proteins selected were validated by Western blot. This technology is thus appropriate for the analysis of scarce samples, such as cell samples from clinical studies with small quantities of protein. Clinical studies are long-term studies that collect biological samples. Cell sampling, cell culture, protein extraction and protein storage are all key steps that must be performed the same way throughout the study, according to appropriate guidelines [19]. The aim of our study was to analyze the proteomic pattern of human macrophages obtained over a 4 year period from blood donors. Our purpose was to simulate a long-term clinical study and assess the applicability and reproducibility of the 2-D DIGE technique for differential proteomic analysis of these scarce samples.

2 Materials and methods

2.1 Isolation and culture of human monocyte-derived macrophages

For a period of 4 years (2002–2006), primary cultures of human monocyte-derived macrophages (MDMs) were prepared as previously described, according to a technique adapted from Boyum *et al.* [20]. Briefly, peripheral blood mononuclear cells from each donor were isolated from 25 mL of blood. Blood was diluted with PBS containing 0.1% w/v EDTA and carefully loaded onto a Ficoll gradient (Eurobio) prepared in a Leucosep tube. After an initial centrifugation step (370 g for 45 min, at room temperature), monocytes were collected at the interface into a new tube and washed with 10 mL PBS, 0.1% w/v EDTA by centrifugation at 370 *g* for 10 min, and then once in PBS alone at 370 *g* for 10 min. Finally, the cell pellet was resuspended in 5 mL RPMI-1640 medium (Invitrogen) containing 0.4% v/v penicillin (10 000 U/mL), 0.4% v/v streptomycin (10 000 mg/mL), 1% w/v glutamine, and 2% w/v sodium pyruvate. The cells were then seeded in 35 mm Primaria dishes (VWR) at a density of 1×10^6 cells *per* dish. After sedimentation for 90 min, the supernatant containing the non-adherent cells was discarded. The adherent cells, consisting of monocytes, were washed three times with 1 mL PBS; 1 mL of fresh medium containing 10% v/v heat-inactivated human serum (Promocell) was then added. The culture medium was changed on day 1 and then every two days. On day 12 of primary culture, MDMs were washed three times with PBS and then incubated for 24 h in serum-free culture medium.

The quality of MDMs in each culture was evaluated as previously described [17] by assessing two markers, one intracellular (endothelin-converting enzyme-1 mRNA, detected by RT-PCR), and one secreted into the culture medium (matrix metralloproteinase-9 activity, determined by gelatin zymography).

2.2 Extraction of intracellular proteins from human macrophages

After 24 h of incubation in serum-free culture medium, MDMs were washed three times with 5 mL of 25 mM Tris, pH 7.4, and scraped into 75 μL of buffer containing 50 mM Tris pH 8.6, 10 mM EDTA, 65 mM DTT, proteinase-inhibitor cocktail (one tablet for 10 mL buffer) (Complete: Roche Diagnostics, Meylan, France), 2000 U/mL deoxyribonuclease I (DNase I) and 2.5 mg/mL ribonuclease A (RNase A) for 10 min at room temperature (Roche Diagnostics), which yielded a concentration of ~60 000 cells/mL. The cell suspension was then homogenized on ice with a minivortex (Polylaboblock), and 7 M urea, 2 M thiourea, 4% w/v CHAPS were added before immediately storage at −20°C.

For one macrophage culture in 2007, proteins were extracted in two different conditions: the first as described in Section 3.1, Fig.1, and the second with the same buffer without addition of DNase I and RNase A.

2.3 Protein-labeling

The 2-D Clean-up kit (GE Healthcare) was used to clean up 100 μg of macrophage protein extracts for DTT elimination. Protein pellets were then resuspended in 20 μL of lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 30 mM Tris, pH 8) until the protein concentration was determined with the Bio-Rad R_CD_C Protein Assay (Bio-Rad, UK) and BSA as the protein standard.

DTT-cleaned samples were reduced and labeled with DIGE saturation dyes as described previously [18]. Briefly, the procedure for the analytical gels consisted in adjusting 5 μg of every cleaned sample to 9 μL with lysis buffer, reducing it by incubation with 1 μL of 2 mM Tris (2-carboxyethyl) phosphine at 37°C for 1 h, adding 2 μL of 2 mM cyanine (Cy) (Cy3 or Cy5) and incubated for 30 min at 37°C. The reaction was stopped by the addition of 12 μL of buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 130 mM DTT and 2% v/v Pharmalyte™ (GE Healthcare), broad range pH 3–10 (GE Healthcare). The procedure for preparative gels consisted in adjusting 500 μg of every sample to 250 μL with lysis buffer, reducing it by incubation with 10 μL of 20 mM Tris (2-carboxyethyl) phosphine for 1 h at 37°C, adding 20 μL of 20 mM Cy3, and incubating for 30 min at 37°C. The reaction was stopped by adding 165.5 μL of the buffer used for the analytical gels.

2.4 2-D DIGE

Before 2-DE, Cy3-labeled samples were mixed with the corresponding Cy5-labeled samples. For the IEF steps, IPG strip (24 cm, linear gradient pH 3–10) were rehydrated with 450 μL of labeled sample mixture in buffer containing 7 M urea, 2 M thiourea and 4% w/v CHAPS in a Protean IEF cell

system (Bio-Rad) for 24 h without applying any current. After rehydration, IEF was performed at 300 V for 3 h, and then at a gradient to 1000 V for 6 h, at a gradient to 8000 V for 3 h and finally at 8000 V for 3 h. After IEF, the IPG strips were incubated for 10 min at room temperature with equilibration buffer containing 6 M urea, 0.1 mM Tris-HCl pH 8, 30% v/v glycerol and 2% w/v SDS, applied to the top of a 12.5% isocratic Laemmli gels, sealed with low-melting temperature agarose (GE Healthcare). SDS-PAGE was run in an Ettan-Daltsix system (GE Healthcare) at 20°C and at a constant voltage of 70 V overnight followed by 300 V until the bromophenol front reached the bottom of the gel. All electrophoresis procedures (labeling, first and second dimension) were performed in the dark.

2.5 Image acquisition and bioinformatic analysis

Gels cast between two low-florescence glass plates were scanned with an Ettan DIGE Imager scanner (GE Healthcare) at excitation/emission wavelengths of 532/580 nm for Cy3 and 633/670 nm for Cy5 to yield images with a pixel size of 100 μm.

Image analysis was performed with Progenesis SameSpots v2.0 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Briefly, after a reference image was chosen, the other 2-D gel images were automatically aligned before manual verification. Spot volumes were then calculated and normalized in each image. In the next step, the appropriate gels were selected for each group. The differences in protein spots were then analyzed. Spots were considered to have significantly different normalized spot volumes if the fold-change was greater than 1.5 and the corresponding *p*-value (one-way ANOVA analysis) was significant. The last step applied multivariate statistics to the selected spots by calculating *q*-values (for the false discovery rate) and power. Principal component analysis was also used to determine whether the data contained any outliers.

2.6 In gel tryptic digestion and peptide extraction

Spots corresponding to proteins differentially expressed between the different groups of samples were manually excised from a preparative gel. Before digestion, spots were washed for 15 min with 100 μL of 50 mM ammonium bicarbonate solution and then twice for 15 min with 50 mM ammonium bicarbonate/50% ACN. They were then dried after adding 100 μL of ACN for 10 min. After discarding the supernatant, tubes were left open for 10 min to complete solvent evaporation. They were then rehydrated with 12 μL of a solution containing 0.025% ProteasMAX™ Surfactant, Trypsin Enhancer (Promega) in 50 mM ammonium bicarbonate and 3 μL of 40 μg/mL Trypsin Gold (Promega) in 50 mM acetic acid. After overnight digestion at 37°C, peptide extraction was carried out in two steps according to the manufacturer's protocol for ProteasMAX™ Surfactant, Trypsin Enhancer. First, after adding 10 μL 0.01% Surfactant solution and shaking for 10 min, we transferred the supernatant into a new tube and added 20 μL of 0.01% Surfactant, 1% TFA solution; after the tube was shaken for 10 min, the supernatant was transferred with the first peptide extraction. Peptides were then purified, desalted with ZipTip C18 tips (Millipore Bedford, MA, USA) according to the manufacturer's protocol, and eluted with 3 μL of 0.1% TFA/50% ACN.

2.7 MALDI-TOF MS and protein identification

For acquisition of the mass spectra of the extracted and desalted peptides, we mixed 0.5 μL of the peptide solution with 0.5 μL of matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid dissolved in 0.1% TFA/50% ACN) on the MALDI-TOF MS target. External calibration was performed with a peptide mixture resulting from the tryptic digest of BSA (0.5 μg/mL). MALDI-TOF MS was then performed with a Voyager DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) equipped with a 337.1 nm nitrogen laser and a delayed extraction facility (125 msec). All spectra were acquired in a positive ion reflector mode under 20 kV voltage, 61% grid. Typically, 300 laser shots were recorded *per* sample. The mass spectra were then calibrated before protein identification by peptide mass fingerprinting, conducted by running the MASCOT web searcher (http://www.matrixscience.com/, Matrix Science, UK) against the NCBInr 20080718 (6 833 826 sequences; 2 363 426 297 residues) with the following parameters: fixed modifications: CyDye-Cy3 (C); Variable modifications: oxidation (M); peptide mass tolerance: ±50 ppm; peptide charge state: 1+; max missed cleavages: 1.

Once the proteins were identified, Gene Ontology analysis of the subcellular localization, molecular functions and biological functions of the proteins in group A and group B was performed, with the freely available internet tool: http://pipe.systemsbiology.net/pipe/#Summary.

3 Results and discussion

3.1 2-D gel pattern of human macrophages

Twenty-six MDM cultures obtained from 2002 to 2006 were randomly selected to undergo 2-D DIGE. Figure 1 presents representative macrophage cultures, all of which showed the same morphology. Only primary cultures corresponding to this morphology and meeting the molecular criteria (endothelin-converting enzyme-1 mRNA expression and matrix metralloproteinase-9 secretion) previously described [17] were used for these 2-D gel profiles. Each protein extract was labeled with either Cy3 or Cy5 saturation dyes. Of the 26 subcultures analyzed by the 2-D DIGE technique, 19 had 2-D DIGE gels of sufficient quality for bioinformatic analysis. Interestingly, the 2-D gel analysis allowed us to classify these macrophage subcultures into two groups according to the presence or

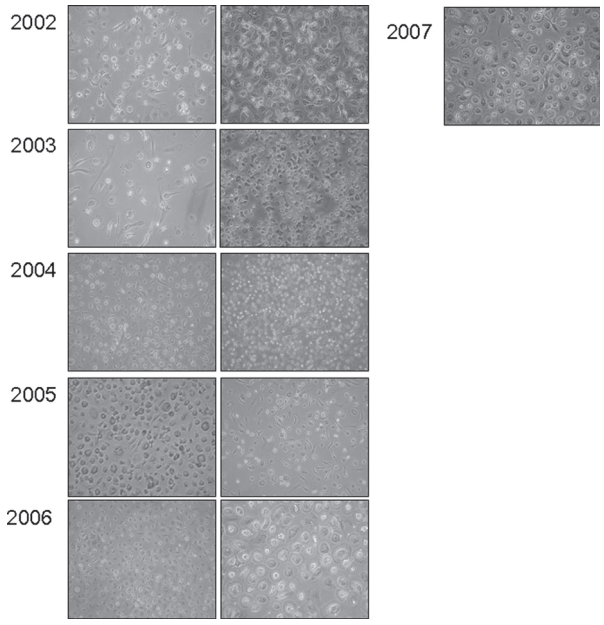

Figure 1. Morphology of human macrophages. Photographs of phase-contrast microscopy were performed on primary culture at day 12. Examples of ten primary cultures of MDM from different donors performed over a 4 year period (from 2002 to 2006) are shown on the left. An example of primary MDM culture in 2007 to evaluate DNase I/RNase A treatment is presented on the right.

absence of five specific spots (Fig. 2). Group A includes the macrophages with the five spots present on the 2-D DIGE gels and group B, the macrophages for which the 2-D DIGE gels lack these five spots (detailed insert in Fig. 2). MALDI-TOF MS made it possible to identify bovine DNase I and annexin A5 as the proteins corresponding to these spots (Supporting Information Table 1). Mass values of DNase I tryptic peptides were also found for polypeptidic spots 3 and 22, identified as annexin A5. These data show that these protein extracts from group A macrophages appears to contain DNase I.

To confirm these findings, we used a culture of macrophages obtained in 2007 (Fig. 1) to extract proteins both with and without DNase I/RNase A treatment. Each sample (with or without DNase I/RNase A) was labeled with either Cy3 or Cy5 to ensure that profiles did not differ due to Cy-labeling. As Fig. 3A shows, each sample produced the same 2-D DIGE profile, whether it was labeled with Cy3 or Cy5. Interestingly, regardless of whether protein extraction did or did not use DNase I/RNase A treatment, all the 2-D DIGE gel images lacked the five spots (white frame in Fig. 3A) that distinguished the macrophages in group A and group B. However, detailed bioinformatic analysis comparing the four representative gel images of these different groups of macrophages revealed that the spots differentially expressed between macrophages treated with and without DNase I/RNase A were the same as between group A and group B (Fig. 3B). Enlargement of five groups of spots (Supporting Information Fig. 1) revealed that the 2-D protein expression profile of

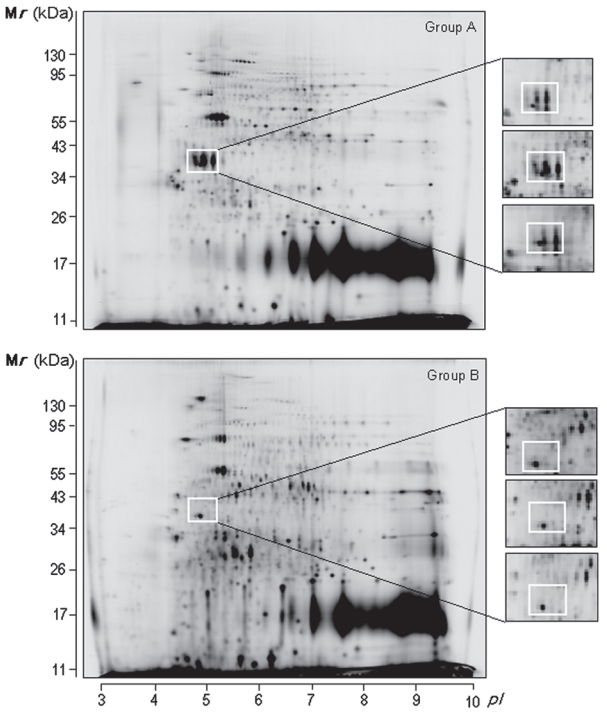

Figure 2. Representative 2-D DIGE gel analysis of intracellular proteome of human macrophages. Macrophage proteins (5 μg) labeled with either Cy3 or Cy5 from cultures of 26 different macrophages were analyzed. Gels were classified according to the presence (group A) or absence (group B) of five specific spots. Detailed insert of area discriminating between group A or group B is presented for three different macrophage' cultures. The positions of molecular weight (*Mr*) standards are indicated on the left, and the p*I* are indicated on the bottom of the gel.

group A was similar to that of the untreated sample and the 2-D profile of group B similar to that of the sample treated with DNase I RNase A. We hypothesize from these findings that the DNase I/RNase A treatment of the macrophages in group A during their protein extraction process, was incomplete, leaving behind protein that results in these spots in the 2-D gel. Moreover, a problem due to a specific batch of DNase I/RNase A is ruled out by the finding that the classification of macrophages into group A or group B does not dependent on the year of the subculture (data not shown). Of the 19 subcultures analyzed, 12 were classified as group A (incomplete DNase I/RNase A treatment) and 7 as group B.

3.2 Differential protein expression between group A and group B macrophages

Because of the difference in the 2-D profiles of group A and group B macrophages, despite the absence of any morphological differences (Fig. 1), we performed a detailed bioinformatic analysis of the 2-D-gels between the two groups. Of 598 spots detected *per* gel (from 24 images of group A and 14 of group B), bioinformatic analysis revealed 104 polypeptide spots differentially expressed between group A and group B,

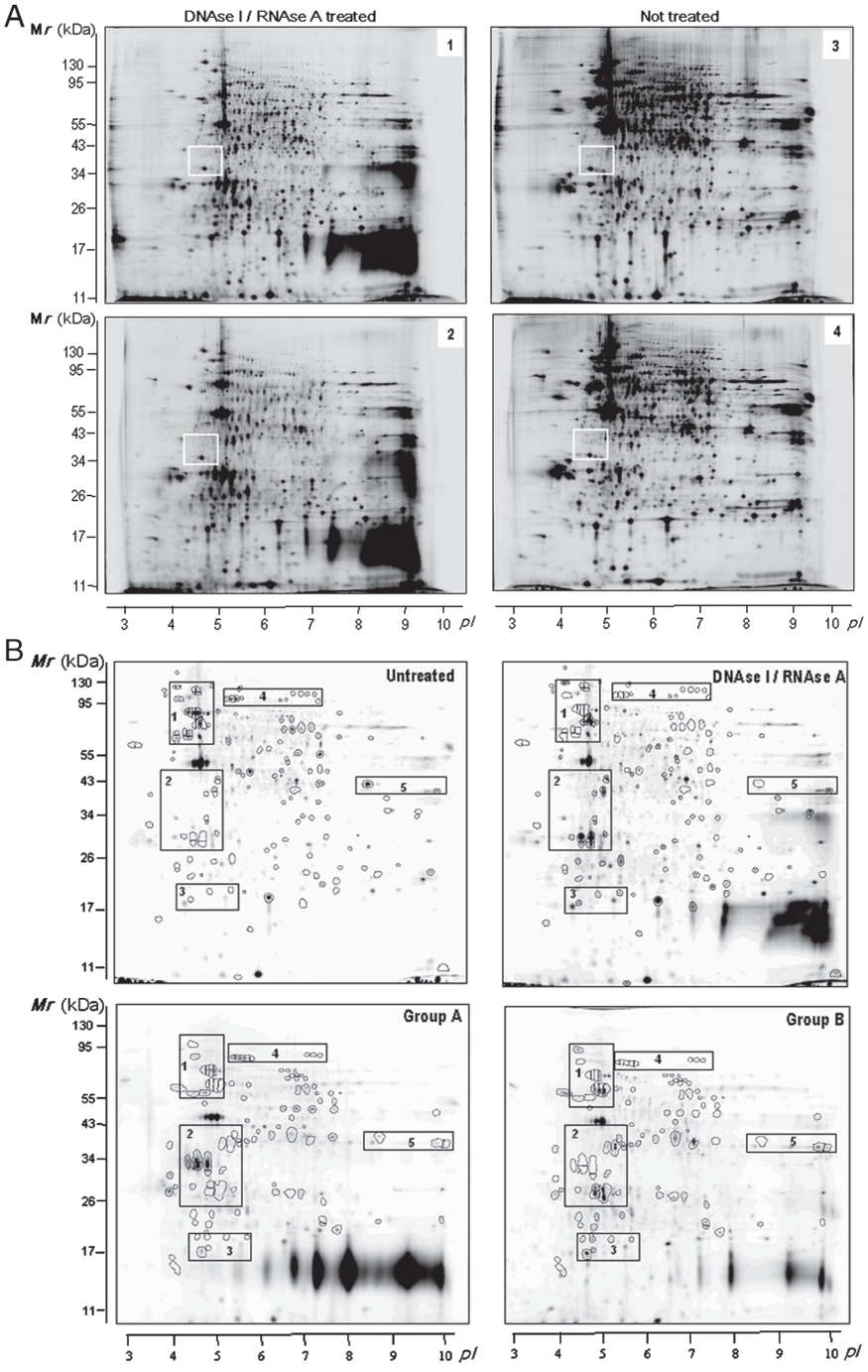

Figure 3. Representative 2-D DIGE gel of intracellular proteome of human macrophages treated by DNase I/RNase A. (A) Representative 2-D DIGE gels of proteins (5 μg) treated with DNase I and RNase A (1, 2) or not treated (3, 4) and labeled with either Cy3 (1, 3) or Cy5 (2, 4). The area corresponding to the five spots discriminating between group A and group B is indicated by a white frame. The positions of molecular weight (*Mr*) standards are indicated on the left and the p*I* are indicated on the bottom of the gels. (B) Bioinformatic comparison of representative 2-D DIGE gels from macrophage proteins treated or not with DNase I/RNase A (upper part) and from macrophage proteins belonging to group A or group B (lower part). Five areas, named 1–5, permitted a detailed bioinformatic comparison. Details of the five areas are presented as supporting online information (Supporting Information Fig. 1). The positions of molecular weight (*Mr*) standards are indicated on the left, and the p*I* are indicated on the bottom of the gels.

located in 20 areas of the 2-D gel (Fig. 4A). Each of these 104 spots was manually validated and selected if its fold-change was greater than 1.5 and its p-value < 0.05. In either case, a q-value < 0.05 was also required for selection. In all, 49 spots were up-regulated and 55 down-regulated in group A compared with group B (Fig. 4B). SameSpots software allowed us to apply principal component analysis to determine whether the selected differential spots were reproducibly detected in all the gels from the same group (data not shown).

3.3 Effect of incomplete DNase I/RNase A treatment on macrophage protein patterns

Of the 104 polypeptide spots found to be differentially expressed between group A and group B, 12 could not be identified (spots 2, 60, 76, 85, 92, 107, 140, 141, 178, 181, 206 and 222) due to the low-intensity signal on MS or the low-probability score. Table 1 reports the identity of the 31 different proteins corresponding to these differentially expressed

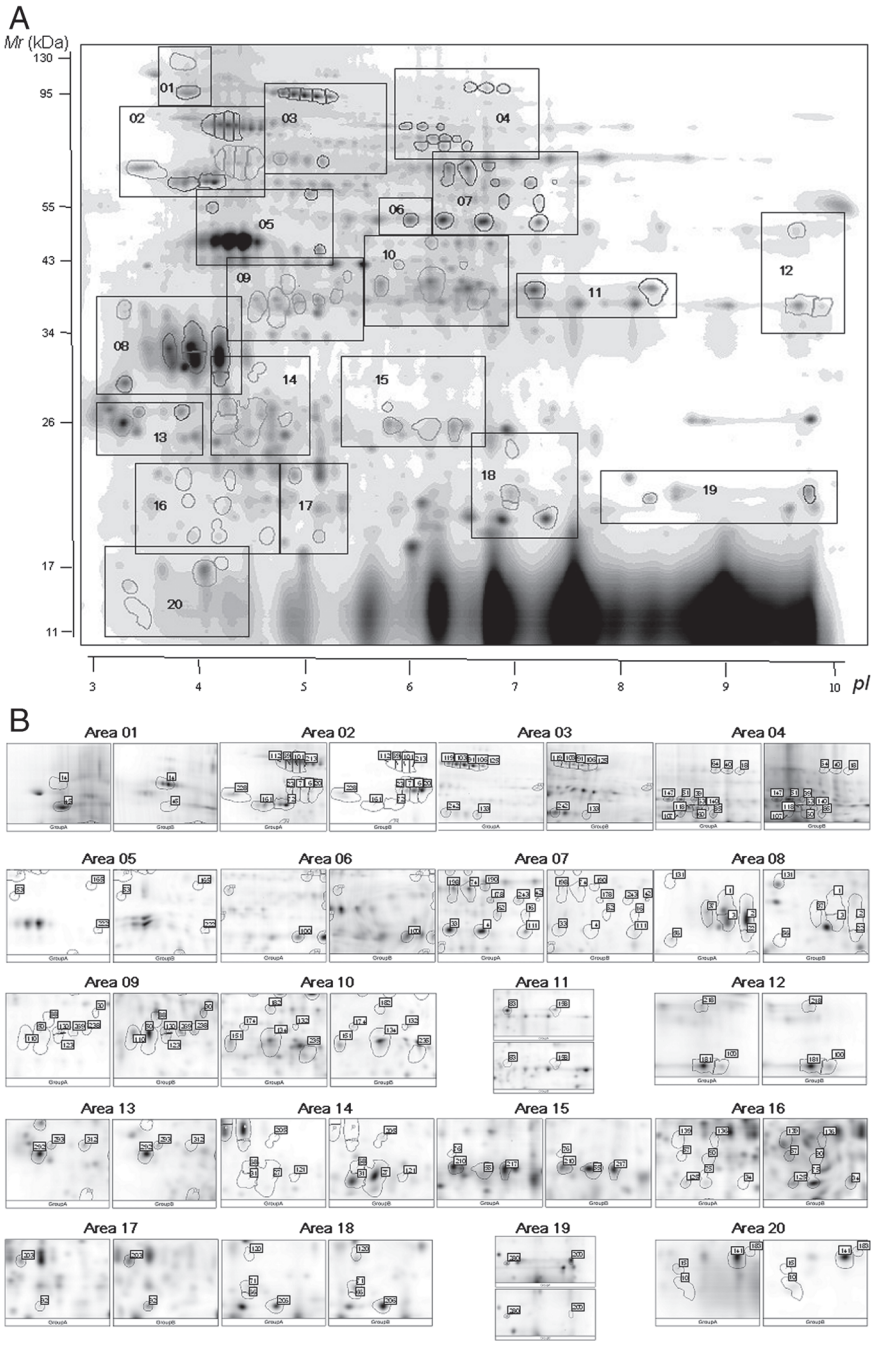

Figure 4. Bioinformatic analysis of 2-D DIGE gels of human macrophages. (A) Representative 2-D DIGE gels analyzed with Progenesis SameSpots v2.0 software. Twenty areas were shown to discriminate between macrophages classified in group A or group B. (B) Detailed of each area showed the spots expressed differentially and statistically significantly between group A and group B.

polypeptide spots. Interestingly, six proteins (annexin A2, phosphoglycerate mutase I, actin cytoplasmic 1, actin cytoplasmic 2, gelsolin and plastin-2) were represented by at least five separate spots. Supporting Information Table 2 presents a detailed MS analysis of the 104 polypeptide spots identified. These proteins were classified according to their biological significance: skeletal system development, gluconeogenesis, glycolysis, anti-apoptosis, cell motion, sensory perception of sound, actin cytoskeleton reorganization, actin filament severing, calcium ion binding, signal transduction, ATP synthesis couple proton transport, cellular response to oxidative stress, cellular chaperone, cell redox homeostasis and GTP binding. The sensitivity and relevance of 2-D DIGE gel technique for proteomic analysis is further demonstrated by our earlier description of most of these proteins [16] in a study that used silver-stained 2-D gels.

Significantly differential expression between groups A and group B was observed for three groups of proteins. The first

Table 1. Detailed list of proteins with statistical differential expression between group A and group B macrophages

Spot number	Accession number	Protein name	Protein function	Subcellular localization	Fold-change (group A *versus* group B)
GO:0001501 Skeletal system development					
34	P07355	Annexin A2	Calcium-regulated membrane-binding protein	Basement membrane	−5.4
83					3.8
90					−3.7
132					−3.1
158					2.8
183					−2.6
66	Q06830	Peroxiredoxin-1	Involved in redox regulation of the cell	Cytoplasm	−4.1
71					−4.1
200					2.4
GO:0006094 Gluconeogenesis					
134	P09467	Fructose-1,6-bisphosphatase 1	Carbohydrate metabolism	Cytosol mitochondrion	-3.1
151					-2.8
GO:0006096 Glycolysis					
4	P06733	α-Enolase	Multifunctional enzyme	Membrane nucleus cytoplasm	10.2
33					5.4
188					2.5
42	P14618	Pyruvate kinase isozymes M1/M2	Glycolytic enzyme	Cytosol	−5.1
190					2.5
55	P18669	Phosphoglycerate mutase 1	Bisphosphoglycerate 2-Phosphatase activity	Cytosol	−4.5
120					−3.2
210					−2.3
217					−2.3
243					2.1
100	P04406	Glyceraldehyde-3-phosphate dehydrogenase	Membrane traficking	Cytoplasm membrane	−3.5
235					−2.2
111	P04075	Fructose-bisphosphate aldolase A	Actin binding	Actin cytoskeleton nucleus	3.4
218					−2.3
GO:0006916 Anti apoptosis					
136*	P09211	Glutathione S-transferase P	Gluthatione transferase activity	Cytoplasm	−3.0
203					−2.4
292	P63104	14-3-3 protein-ζ/δ	Adaptor protein in signaling pathways	Cytoplasm	−1.8
GO:0006928 Cell motion					
10	P08670	Vimentin	Class-III intermediate filaments	Cytoplasm	−8.0
15					−6.8
72					4.1
165*					2.7
27*	P60709	Actin, cytoplasmic 1	Involved in various types of cell motility	Cytoskeleton cytoplasm	−5.7
30*	P63261	Actin, cytoplasmic 2			−5.5
31*					−5.5
50*					−4.7
57*					−4.5
68*					−4.1
88*					−3.7
110*					−3.4
121*					−3.2
123*					−3.2
130*					−3.2
136*					−3.0
139*					−3.0
205*					−2.4

Table 1. Continued

Spot number	Accession number	Protein name	Protein function	Subcellular localization	Fold-change (group A *versus* group B)
238*					−2.1
269*					−1.9
39	P26038	Moesin	Involved in connections of major cytoskeletal structures to the plasma membrane	Cytoplasm cytoskeleton membrane	5.3
51					4.5
147					2.9
56	P06753	Tropomyosin α-3 chain	Binds to actin filaments	Cytoplasm cytoskeleton	4.5
293*					−1.8
161	P07437	Tubulin-β-chain	Major constituent of macrotubules	Microtubule	2.8
165*	P61158	Actin-related protein 3	Involved in regulation of actin polymerisation	Cytoplasm	2.7
312	P52565	Rho GDP-dissociation inhibitor 1	Regulates the GDP/GTP exchange reactions of the Rho proteins	Cytoplasm cytoskeleton	1.7
GO:0007605 Sensory perception of sound					
63	O75083	WD repeat-containing protein 1	Involved in disassembly of Actin filaments	Cytoplasm cytoskeleton	−4.0
118					−2.8
GO:0030036 Actin cytoskeleton organization					
14	P21333	Filamin-A	Links actin filament to memebrane glycoproteins	Cytoplasm	−7.0
GO:0051014 Actin filament severing					
91	P06396	Gelsolin	Actin-modulating protein that binds to the barbed ends of actin	Cytoplasm	3.7
103					3.5
106					3.4
119					3.2
125					3.2
GO:0005509 Calcium ion binding					
6	P13796	Plastin-2	ABP	Cytoplasm	−9.8
7					−9.5
20					−6.4
23					−6.3
69					4.1
101					3.5
112					3.3
213					2.3
242					−2.1
GO:0007165 Signal transduction					
75	P61224	Ras-related protein Rap-1b	Belongs to the small GTPase superfamily	Cytoplasm membrane	−4.0
152					−2.8
GO:0015986 ATP synthesis coupled proton transport					
53	P06576	ATP synthase subunit-β	Produces ATP from ADP	Mitochondria	4.5
GO:0034599 Cellular response to oxidative stress					
16	P52209	6-Phosphogluconate dehydrogenase	Carbohydrate degradation	Cytoplasm	6.8
62					4.3
182	P11413	Glucose-6-phosphate-1-dehydrogenase	Main producer of NADPH reducing power	Cytosol	−2.6
198					2.4
74					4.1
280	P30043	Flavin reductase	Possible role in protecting cells from oxidative damage	Cytoplasm	−1.8

Table 1. Continued

Spot number	Accession number	Protein name	Protein function	Subcellular localization	Fold-change (group A *versus* group B)
GO:0034619 Cellular chaperone					
45	P07900	Heat shock protein HSP 90-α	Molecular chaperone	Cytosol	4.8
131					−3.2
174					−2.7
GO:0045454 Cell redox homeostasis					
133	P30101	Protein disulfide-isomerase A3	Catalyze the rearrangement of disulfide bonds	Endoplasmic reticulum	3.1
228	P07237	Protein disulfide-isomerase	Catalyze the formation, breakage and rearrangement of disulfide bonds	Endoplasmic reticulum	−2.2
GO:0005525 GTP binding					
18	P13639	Elongation factor 2	Promotes the GTP-dependent translocation	Cytoplasm	6.7
40					5.1
64					4.2

Spots with *p*- (ANOVA) < 0.05 and *q*-value (probability to false discovery rate) < 0.05 were selected to be identified by MALDI-TOF MS. Monoisotopic peptides masses were searched for NCBI protein databases using MASCOT search engine (http://www.matrixscience.com/). Assignments were made according to UniProtKB/SwissProt Release 15.1/57.1 of April 14, 2009 (462,764 entries). Theo. = theoretical. Exp. = experimental. Asterisk for these spots, peptide mass fingerprint search gave more than one protein with a significant probability-based mowse score, all possible results are shown in the table.

group comprised the proteins involved in glycolysis (α-enolase, pyruvate kinase isozymes M1/M2, phosphoglycerate mutase 1, glyceraldehyde-3-phosphate dehydrogenase and fructose-bisphosphate aldolase A), and the second group those involved in cellular response to oxidative stress (6-phosphogluconate dehydrogenase, glucose-6-phosphate-1-dehydrogenase and flavin reductase). The third group, interestingly, comprised 13 proteins involved in cell motion and actin reorganization: vimentin, actin cytoplasmic 1, actin cytoplasmic 2, moesin, tropomyosin α-3 chain, tubulin β-chain, actin-related protein 3, rho GDP-dissociation inhibitor 1, WD repeat-containing protein 1, filamin-A, gelsolin, plastin-2 and elongation factor 2. This group accounts for one third of the proteins identified: four of them were down-regulated (filamin-A, actin cytoplasmic 1, actin cytoplasmic 2 and WD repeat-containing protein 1) and six up-regulated (gelsolin, moesin, tubulin β-chain, actin-related protein 3, rho GDP-dissociation inhibitor 1 and elongation factor 2) in group A compared with group B. Three proteins (plastin-2, vimentin and tropomyosin α-3 chain) appeared in spots that were both up and down-regulated in group A compared with group B, a situation that suggests a possible differential post-translational modification or protein cleavages. Except tubulin β-chain, whose presence is characteristic of microtubules only, twelve proteins are located in the cytoplasm and 6 of them in the cytoskeleton (actin cytoplasmic 1, actin cytoplasmic 2, moesin, tropomyosin α-3 chain, rho GDP-dissociation inhibitor 1 and WD repeat-containing protein 1). Interestingly, we also observed a shift of cytoplasmic proteins between group A (14%) and group B (24%) and an inverse shift of cytoskeleton proteins (27 and 14%, respectively). These subcellular localization are consistent with previous 2-D maps of the human macrophage proteome [16], where the protein pattern seemed to be that of group B (complete DNase I treatment).

Interestingly, DNase I has been described not only as having nuclease activity, but also as playing a role in the regulation of actin polymerization; indeed actin is the naturally occurring inhibitor of DNase I [21]. DNase I was initially though to have greater affinity for monomeric (G-actin) than for filamentous (F-actin) actin [22], but a recent study reported similar dissociation constants of DNase for both G-actin and F-actin [23]. However, the biological significance of the interaction between actin and DNase I remains unknown. Several studies have examined the interaction of the actin:DNase I complex with other actin-binding proteins (ABPs) [24–26]. DNase I affinity chromatography has captured actin complex with other ABPs [27], and the commonly used DNase I inhibition assay has been used to quantify monomeric and polymeric actin [28]. Based on our results, treatment of protein samples with DNase I clearly appears to explain why the main differences in protein expression profiles between group A and group B are for proteins involved in actin reorganization and cell motion, including actin cytoplasmic 1 and actin cytoplasmic 2.

Our data clearly showed that DNase treatment, in the expectation of nuclease activity, can be a source of bias because of the strong interaction between DNase I and actin and the possible role of DNase I in the regulation of actin cytoskeleton. At a minimum, this finding is pertinent to long-term studies and to samples that are cell protein extracts. We strongly recommend the use of alternative techniques, such as sonication or precipitation, to remove nucleic acids from protein samples. Another potential recently proposed technique is a two-phase extraction of

DNA in chloroform/phenol/isoamyl alcohol to remove DNA from *E. coli* extracts [29].

4 Concluding remarks

The use of DNase I for the removal of nucleic acids from protein samples must be avoided, for it can engender bias in the analysis of protein expression patterns in proteomic studies. Not only does DNase I have nuclease activity, but it has also been reported to be involved in actin cytoskeleton regulation because of its high affinity for actin. During our 4 year 2-D DIGE study of the protein expression profiles of human macrophages, we found that DNase I treatment produced changes in their proteome profiles. The proteins mainly affected by this treatment were those involved in cell motion and actin cytoskeleton reorganization.

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The authors have declared no conflict of interest.

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3.1.2.2. Supporting information and additional data on proteomic analysis between Group A and Group B of macrophages

Supplementary information quoted in the article is presented in Supplementary Table 1A, Supplementary Table 1B, Supplementary Figure 1, and Supplementary Table 2. Additionally, Supplementary Table 3 presents statistical values of differentially expressed spots between groups A and B of macrophages selected during bioinformatic analysis with SameSpots software.

Supplementary Table 1A. Detailed mass spectrometry analysis of the 5 differential spots between Group A and Group B macrophages.

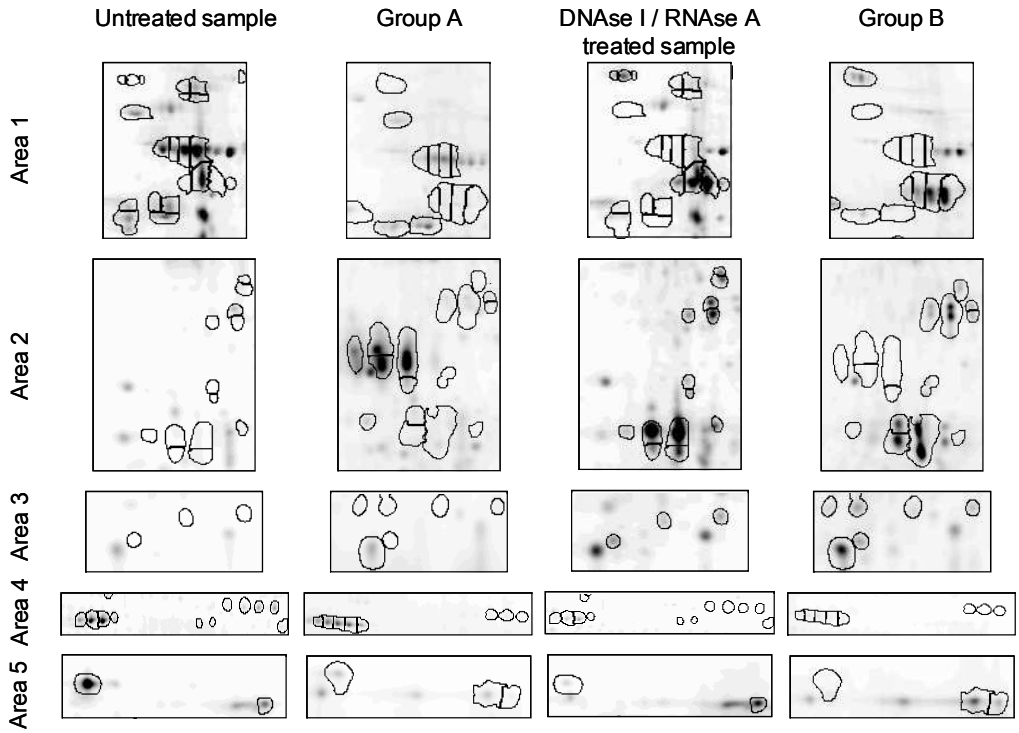
Spot Number	Accession number	Protein name	Theo. *Mr* (kDa)	Exp. *Mr* (kDa)	Theo. *pI*	Exp. *pI*	Number of matched peptides/total peptides	Sequence coverage (%)	MASCOT Score (NCBI)
1	P00639	Deoxyribonuclease-1 (Bovine)	31.3	34	5.34	4.7	6/13	20	83
37				34		4.5	5/9	17	74
3	P08758	Annexin A5	36.6	31	4.94	4.8	11/24	39	132
22				31		4.9	7/30	29	68
2*	-	Non-significant identification	-	34	-	4.9	-	-	-

*For spot number 2, deoxyribonuclease-1 (Bovine) was the first possible protein with a non-significant MASCOT Score of 59.

Supplementary Table 1B. Detailed mass spectrometry analysis for spots 1, 3, 22 and 37.

Spot number	Protein name	m/z submitted	MH^+ matched	Start-End	Peptide sequence
1	Deoxyribonuclease-1 (Bovine)	804.4737	804.4653	25-31	(K)IAAFNIR(T)
		925.4871	925.5029	64-72	(R)DSHLVAVGK(L)
		1197.6678	1197.6706	102-110	(R)YLFLFRPNK(V)
		1247.6870	1247.6921	54-63	(R)YDIVLIQEVR(D)
		1403.7933	1403.7932	53-63	(R)RYDIVLIQEVR(D)
		2721.3174	2721.3082	73-95	(K)LLDYLNQDDPNTYHYVVSEPLGR(N)
3	Annexin A5	954.5224	954.5335	194-201	(K)FITIFGTR(S)
		1014.492	1014.5069	90-97	(R)LYDAYELK(H)
		1106.5687	1106.5768	227-285	(R)SEIDLFNIR(K)
		1156.6683	1156.6798	152-161	(R)MLVVLLQANR(D)
		1340.5990	1340.6045	7-18	(R)GTVTDFPGFDER(A)

		1613.9075	1613.9036	228-242	(R)ETSGNLEQLLLAVVK(S)
		1704.8941	1704.8941	30-45	(K)GLGTDEESILTLLTSR(S)
		1799.9034	1799.889	187-201	(K)WGTDEEKFITIFGTR(S)
		1802.8693	1802.8556	213-227	(K)YMTISGFQIEETIDR(E)
		1818.8524	1818.8506	213-227	(K)YMTISGFQIEETIDR(E) Oxidation (M)
		2888.2482	2888.2308	127-151	(K)QVYEEEYGSSLEDDVVGDTSGYYQR(M)
22	Annexin A5	954.5478	954.5335	193-200	(K)FITIFGTR(S)
		1037.5558	1037.5553	70-78	(K)SELTGKFQK(L)
		1106.5965	1106.5768	276-284	(R)SEIDLFNIR(K)
		1340.6343	1340.6045	6-17	(R)GTVTDFPGFDER(A)
		1704.9355	1704.8941	29-44	(K)GLGTDEESILTLLTSR(S)
		1802.8981	1802.8556	212-226	(K)YMTISGFQIEETIDR(E)
		2888.3312	2888.2308	126-150	(K)QVYEEEYGSSLEDDVVGDTSGYYQR(M)
37	Deoxyribonuclease-1 (Bovine)	804.4600	804.4653	25-31	(K)IAAFNIR(T)
		1197.6658	1197.6706	102-110	(R)YLFLFRPNK(V)
		1247.6820	1247.6921	54-63	(R)YDIVLIQEVR(D)
		1403.7888	1403.7932	53-63	(R)RYDIVLIQEVR(D)
		2721.3247	2721.3082	73-95	(K)LLDYLNQDDPNTYHYVVSEPLGR(N)



Supplementary Figure 1: Bioinformatic comparison of representative 2D-DIGE gels of macrophage proteins treated or not with DNase I and RNase A and of macrophage proteins from Group A and Group B. Enlargement of the five areas where spots with similar expression profiles in both analysis are localized. The protein pattern of Group A macrophages corresponds that of untreated MDM samples and the protein profile of Group B of macrophages to protein profile of MDM sample treated DNase I/RNase A.

Supplementary Table 2. Detailed mass spectrometry analysis of polypeptide spots selected for a differential expression between Group A and Group B as indicated in Figure 4B.

Spot Number	Accession number	Protein name	Theo. *Mr* (kDa)	Exp. *Mr* (kDa)	Theo. *pI*	Exp. *pI*	Number of matched peptides/ total peptides	Sequence coverage (%)	MASCOT Score (NCBI)
4				50		6.9	11/26	29	100
33	P06733	α-Enolase	51.2	50	7.0	6.7	13/24	41	128
188				50		6.4	13/25	35	129
6				70		5.0	18/30	34	185
7				70		4.9	16/32	31	157
20				70		5.1	12/28	23	106
23				70		4.9	12/26	24	112
69	P13796	Plastin-2	70.3	76	5.2	4.9	17/40	30	146
101				78		5.0	17/40	30	146
112				78		4.9	17/40	30	146
213				76		5.0	17/40	30	146
242				70		5.3	11/36	22	95
10				17		4.1	5/8	22	73
15	P08670	Vimentin	53.6	19	5.0	4.0	6/12	26	82
72				59		4.8	8/12	22	101
165*				56		5.6	10/40	25	73

14	P21333	Filamin-A	280.7	115	5.7	4.6	16/29	11	102
16	P52209	6-Phosphogluconate dehydrogenase	53.1	55	6.8	7.4	11/40	26	75
62				55		7.2	14/40	29	101
18	P13639	Elongation factor 2	106.7	94	6.4	7.2	13/40	16	85
40				94		7.1	13/30	16	106
64				94		7.0	18/40	22	135
27*	P60709	Actin, cytoplasmic 1	41.7	27	5.3	5.0	8/13	26	102
30*				41		5.7	12/30	39	122
31*				27		4.9	8/14	26	99
50*				41		5.1	11/21	35	119
57*				23		4.6	6/15	23	72
68*				-		-	6/14	17	66
88*				42		5.2	9/18	34	124
110*	P63261	Actin, cytoplasmic 2	41.8	41	5.3	5.0	11/26	35	116
121*				27		5.3	9/18	26	103
123*				41		5.3	9/17	34	115
130*				42		5.4	10/20	35	130
136*				24		4.8	9/40	37	74
139*				24		4.5	7/27	26	70
205*				32		5.1	8/11	31	112
238*				40		5.7	9/30	34	101

269*				41		5.5	11/22	35	130
34	P07355	Annexin A2	38.6	20	7.6	5.0	7/33	26	66
83				42		7.4	18/36	52	182
90				23		4.8	10/38	29	91
132				42		7.4	19/40	47	171
158				41		8.1	10/27	34	106
183				20		4.8	11/20	34	140
39	P26038	Moesin	69.1	83	6.1	6.7	11/22	19	130
51				83		6.6	13/20	22	139
147				83		6.4	12/17	21	150
42	P14618	Pyruvate kinase isozymes M1/M2	57.9	70	7.9	7.5	17/40	31	142
190				68		7.1	14/40	28	105
45	P07900	Heat shock protein HSP 90-α	89.3	90	4.9	4.6	18/40	23	151
131				40		3.7	6/8	8	74
174				42		6.6	13/40	21	95
53	P06576	ATP synthase subunit β	56.5	55	5.2	4.7	15/19	46	231
55	P18669	Phosphoglycerate mutase 1	28.8	26	6.7	6.5	9/23	46	123
120				25		7.2	8/40	46	80
210				26		6.2	9/36	46	104
217				26		6.7	10/40	47	104

243				59		7.3	9/36	37	97
56	P06753	Tropomyosin alpha-3 chain	29.0	30	4.7	4.1	10/24	28	107
293*				27		4.2	13/40	37	117
63	O75083	WD repeat-containing protein 1	66.2	78	6.2	6.7	11/40	26	93
118				78		6.6	13/40	31	116
66	Q06830	Peroxiredoxin-1	22.1	23	8.3	7.1	8/25	45	113
71				23		7.1	8/25	45	113
200				22		9.5	12/38	56	152
74	P11413	Glucose-6-phosphate 1-dehydrogenase	59.2	65	6.4	6.9	14/21	32	177
182				43		6.7	11/40	24	87
198				65		6.7	16/25	33	182
75	P61224	Ras-related protein Rap-1b	20.8	22	5.6	4.8	6/21	45	78
152				22		4.6	5/19	27	70
91	P06396	Gelsolin	80.6	87	5.6	5.5	17/34	28	192
103				87		5.5	13/20	22	157
106				87		5.6	18/24	30	223
119				87		5.4	16/40	27	146
125				87		5.7	15/28	23	160
100	P04406	Glyceraldehyde-3-phosphate dehydrogenase	36.1	41	8.6	9.8	6/15	24	78
235				41		7.5	6/13	25	80
111	P04075	Fructose-	39.4	50	8.3	7.5	11/40	30	99

218		bisphosphate aldolase A		50		9.5	13/40	39	124
133	P30101	Protein disulfide-isomerase A3	56.8	65	6.0	5.7	18/40	36	160
134	P09467	Fructose-1,6-bisphosphatase 1	36.8	42	6.5	6.9	7/24	25	72
151				42		6.5	7/21	25	80
136	P09211	Glutathione S-transferase P	23.3	24	5.4	4.8	7/40	42	76
203				24		5.4	7/30	42	83
161	P07437	Tubulin beta chain	49.7	61	4.8	4.5	16/32	40	158
165*	P61158	Actin-related protein 3	47.4	56	5.6	5.6	9/40	28	70
228	P07237	Protein disulfide-isomerase	57.1	70	4.7	4.2	13/27	30	143
280	P30043	Flavin reductase	22.1	23	7.1	8.3	9/16	64	128
292	P63104	14-3-3 protein ζ/δ	27.7	26	4.7	4.0	7/16	28	81
312	P52565	Rho GDP-dissociation inhibitor 1	23.2	27	5.0	4.7	8/28	34	90
60	-	Not identified	-	70	-	6.7	-	-	-
76				27		6.2			
85				70		6.9			
92				20		5.7			
107				70		6.6			
140				78		6.8			

141	19	4.7
178	59	7.1
181	41	9.5
206	22	7.5
222	44	5.5

Monoisotopic peptides masses were searched for NCBI protein databases using MASCOT search engine (http://www.matrixscience.com/). Assignments were made according to UniProtKB/SwissProt Release 15.1 / 57.1 of April 14, 2009 (462,764 entries). Theo. = theoretical. Exp. = experimental. Asterisk for these spots, peptide mass fingerprint search gave more than one protein with a significant probability based mowse score, all possible results are shown in the table.

Supplementary Table 3. Statistical values of selected spots during the analysis with SameSpots software.

Spot	p (ANOVA)	q Value	Power	Fold Change (Group A *vs.* Group B)	Spot	p (ANOVA)	q Value	Power	Fold Change (Group A *vs.* Group B)
1	1.30E-11	2.88E-10	1	23.3	107	2.27E-05	2.96E-05	1	3.4
2	2.06E-11	3.98E-10	1	15.5	110	7.06E-12	1.83E-10	1	-3.4
3	1.48E-09	1.16E-08	1	11.6	111	6.48E-06	1.01E-05	1	3.4
4	7.71E-13	2.39E-11	1	10.2	112	2.74E-06	5.00E-06	1	3.3
6	1.10E-14	9.80E-13	1	-9.8	118	3.26E-05	3.40E-05	1	3.2
7	1.25E-14	9.80E-13	1	-9.5	119	1.16E-05	1.82E-05	1	3.2
10	4.56E-07	1.31E-06	1	-8.0	120	8.45E-07	1.99E-06	1	-3.2
14	4.53E-09	2.72E-08	1	-7.0	121	5.36E-05	5.60E-05	1	-3.2
15	4.08E-05	4.27E-05	0.999	-6.8	123	1.62E-06	3.39E-06	1	-3.2
16	1.20E-08	6.51E-08	1	6.8	125	5.68E-05	5.93E-05	0.999	3.2
18	2.00E-07	6.27E-07	1	6.7	130	1.25E-08	6.51E-08	1	-3.2
20	5.35E-10	5.17E-09	1	-6.4	131	1.60E-05	2.26E-05	1	-3.2
22	1.00E-07	3.66E-07	1	6.3	132	4.35E-05	4.55E-05	1	-3.1
23	1.42E-09	1.16E-08	1	-6.3	133	2.46E-05	3.21E-05	1	3.1
27	6.49E-13	2.39E-11	1	-5.7	134	6.72E-07	1.72E-06	1	-3.1
30	1.39E-09	1.16E-08	1	-5.5	136	1.27E-06	2.99E-06	1	-3.0
31	2.48E-09	1.56E-08	1	-5.5	139	4.83E-08	1.99E-07	1	-3.0
33	1.54E-09	1.16E-08	1	5.4	140	4.62E-04	3.62E-04	0.995	2.9
34	2.82E-10	3.96E-09	1	-5.4	141	5.77E-04	4.52E-04	0.992	-2.9
37	1.30E-05	2.03E-05	1	5.3	147	1.69E-04	1.32E-04	0.998	2.9
39	2.47E-07	7.74E-07	1	5.3	151	1.06E-05	1.66E-05	1	-2.8
40	2.16E-08	1.01E-07	1	5.1	152	9.56E-07	2.25E-06	1	-2.8
42	1.23E-06	2.88E-06	1	-5.1	158	6.36E-05	6.64E-05	0.999	2.8
45	1.35E-06	3.16E-06	1	4.8	161	9.97E-05	1.04E-04	1	2.8
50	3.45E-13	1.79E-11	1	-4.7	165	1.70E-04	1.33E-04	0.999	2.7
51	6.03E-07	1.58E-06	1	4.5	174	4.94E-05	5.16E-05	1	-2.7
53	2.96E-10	3.96E-09	1	4.5	178	2.34E-05	3.06E-05	1	2.6
55	3.61E-10	3.96E-09	1	-4.5	181	3.75E-06	6.86E-06	1	-2.6
56	4.30E-10	4.38E-09	1	4.5	182	1.99E-07	6.27E-07	1	-2.6
57	6.06E-08	2.37E-07	1	-4.5	183	2.53E-05	3.28E-05	1	-2.6
60	2.10E-05	2.75E-05	1	4.4	188	2.31E-05	3.02E-05	1	2.5
62	4.20E-06	7.68E-06	1	4.3	190	1.39E-03	7.24E-04	0.985	2.5
63	3.81E-06	6.96E-06	1	4.2	198	1.58E-04	1.27E-04	0.998	2.4
64	8.33E-07	1.96E-06	1	4.2	200	2.34E-04	1.83E-04	0.997	2.4
66	8.88E-08	3.25E-07	1	-4.1	203	3.10E-04	2.43E-04	0.993	-2.4
68	2.28E-09	1.56E-08	1	-4.1	205	1.13E-04	1.18E-04	0.999	-2.4

69	6.91E-06	1.08E-05	1	4.1	206	1.46E-05	2.26E-05	1	-2.4
71	1.35E-08	6.68E-08	1	-4.1	210	2.17E-05	2.83E-05	1	-2.3
72	5.37E-06	8.52E-06	1	4.1	213	2.60E-03	1.36E-03	0.955	2.3
74	4.59E-07	1.32E-06	1	4.1	217	1.25E-04	1.27E-04	0.998	-2.3
75	2.32E-09	1.56E-08	1	-4.0	218	1.83E-04	1.44E-04	0.998	-2.3
76	4.68E-04	3.67E-04	0.984	-4.0	222	7.71E-04	4.83E-04	0.981	2.3
83	5.53E-07	1.45E-06	1	3.8	228	2.18E-04	1.71E-04	0.995	-2.2
85	2.26E-05	2.95E-05	1	3.8	235	1.43E-04	1.27E-04	0.999	-2.2
88	3.43E-10	3.96E-09	1	-3.7	238	2.29E-05	3.00E-05	1	-2.1
90	2.74E-08	1.21E-07	1	-3.7	242	1.96E-05	2.57E-05	1	-2.1
91	7.32E-07	1.72E-06	1	3.7	243	1.12E-03	5.87E-04	0.985	2.1
92	4.89E-06	8.52E-06	1	-3.7	269	4.24E-04	3.32E-04	0.989	-1.9
100	1.52E-06	3.18E-06	1	-3.5	280	7.12E-04	4.83E-04	0.984	-1.8
101	2.72E-05	3.28E-05	1	3.5	292	2.75E-04	2.15E-04	0.995	-1.8
103	4.71E-06	8.52E-06	1	3.5	293	7.04E-04	4.83E-04	0.994	-1.8
106	6.67E-06	1.05E-05	1	3.4	312	5.98E-04	4.69E-04	0.982	1.7

Fold Change is shown as a mean of normalized volume of the same spot in gels belonging to Group A compared to the corresponding values of Group B.

3.1.2.3. Differential protein expression between AAA and non-AAA macrophage samples

Regarding the disease status classification of the samples, the proteomic profiles of 2D-DIGE gels of AAA and non-AAA samples were compared following two different strategies: with and without consideration of the classification in Group A and Group B proteomic profile. During bioinformatic analysis, the same parameters and criteria described above for comparison between group A and group B were applied.

When proteomic profile classification was not taken into account, corresponding images of 2D-DIGE gels of macrophage protein extracts of 10 AAA patients (10 Cy3 images + 10 Cy5 images) were compared to corresponding images of 9 non-AAA patients (9 Cy3 images + 9 Cy5 images). After manual validation, bioinformatic analysis revealed that none of the 598 spots detected *per* image was significantly differentially expressed between both groups of macrophage samples, probably due to the strong influence of the technical bias.

When gels were analyzed within group A of proteome profiles, corresponding images of 2D-DIGE gels of macrophage protein extracts of 7 AAA patients (7 Cy3 images + 7 Cy5 images) were compared to corresponding images of 5 non-AAA patients (5 Cy3 images + 5 Cy5 images). After

manual validation, bioinformatic analysis revealed that none of the 598 spots detected *per* image was significantly differentially expressed between both groups of macrophage samples.

When gels were analyzed within group B of proteome profiles, corresponding images of 2D-DIGE gels of macrophage protein extracts of 3 AAA patients (3 Cy3 images + 3 Cy5 images) were compared to corresponding images of 4 non-AAA patients (4 Cy3 images + 4 Cy5 images). Of 598 spots detected *per* image, after manual validation, bioinformatic analysis revealed 2 spots differentially under-expressed in AAA compared to non-AAA macrophage samples (Figure 1). Table 1 shows statistical values of these spots calculated by SameSpot software during differential proteomic analysis.

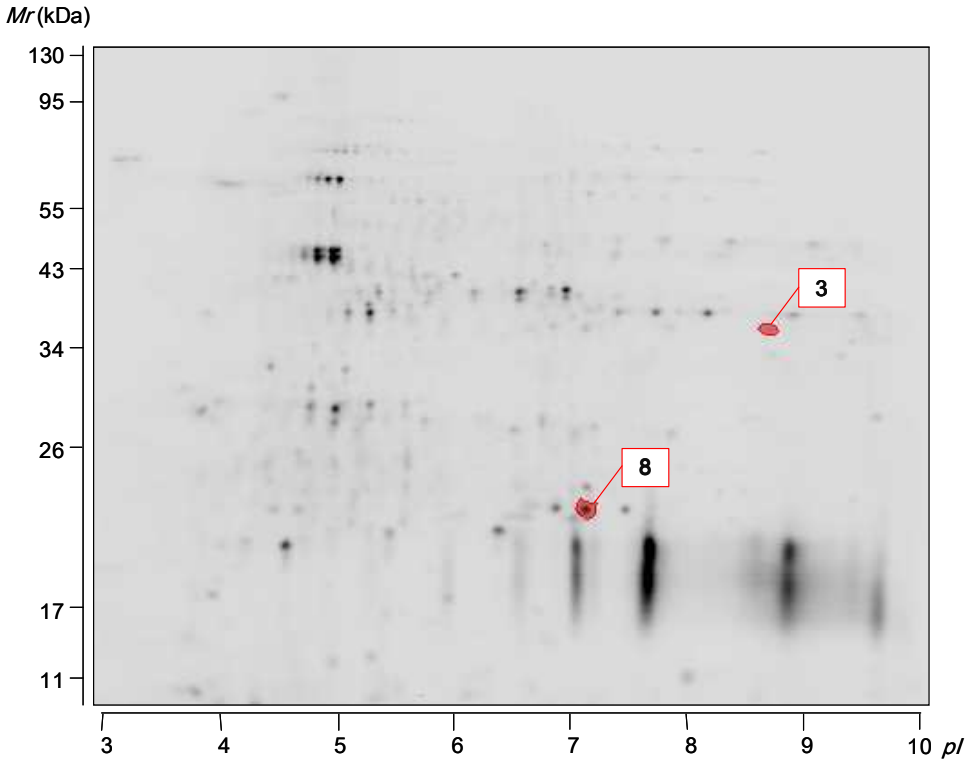


Figure 1. Bioinformatic analysis of 2D-DIGE gels of human macrophages included in group B proteome profile. Representative image of 2D-DIGE gel performed with the macrophage protein extract of an AAA patient. Polypeptide spots highlighted in red were differentially expressed between AAA and non-AAA macrophage samples. The positions of molecular weight (M*r*) standards are indicated on the left, and the *pI* are indicated on the bottom of the gel.

MALDI-TOF mass spectrometry analysis of the two selected spots allowed identification of the following proteins: annexin A2 and manganese superoxide dismutase (MnSOD) (Table 2).

Table 1. Statistical values of differentially expressed spots between AAA and non-AAA macrophage protein extracts belonging to group B proteome profile

Spot	p (ANOVA)	q Value	Power	Fold Change (AAA *vs.* non-AAA)
3	0.00095	0.22	0.954	-3.8
8	0.0043	0.30	0.842	-2.6

Fold Change is shown as a mean of normalized volume of the same spot in gels of AAA patients compared to the corresponding values of non-AAA patients.

Table 2. Detailed mass spectrometry analysis of polypeptide spots selected for differential expression between AAA and non-AAA macrophage samples belonging to group B proteome profile.

Spot Number	Accession number	Protein name	Theo. *Mr*/ Exp. *Mr* (kDa)	Theo. *pI*/ Exp. *pI*	Number of matched peptides/ total peptides	Sequence coverage (%)	MASCOT Score (NCBI)
3	P07355	Annexin A2	41.2/ 36	7.57/ 8.7	17/31	45	173
8	P04179	Manganese superoxide dismutase	26.7/ 22	8.35/ 7.1	5/15	22	70

Monoisotopic peptides masses were searched for NCBI protein databases using MASCOT search engine (http://www.matrixscience.com/). Assignments were made according to UniProtKB/SwissProt Release 15.1 / 57.1 of April 14, 2009 (462,764 entries). Theo. = theoretical. Exp. = experimental.

Group B of 2D-DIGE proteomic profile of macrophage protein extracts corresponded to samples in which DNAse I treatment was complete, giving rise to an unexpected modified protein profile in comparison to the untreated samples. Of the two differentially expressed proteins between macrophage samples of AAA patients and non-AAA patients belonging to group B, one protein, Annexin A2, a secreted protein that interacts non-covalently with membrane proteins and is involved in skeletal system development (GO: 0001501), already appeared to be differentially expressed between group A and group B of proteomic profiles. Indeed, 4 spots showed up-regulation of

Annexin A2 in group B compared to group A, and 2 spots showed down-regulation of Annexin A2 in group B compared to group A. This situation suggests possible differential post-translational modifications or protein cleavages. Consequently, since expression and/or post-translational processing of Annexin A2 was already modified during DNAse I treatment of the samples, the results obtained regarding patient status are irrelevant because we cannot make sure that DNAse I treatment did not affect this specific spot too.

MnSOD belongs to the superoxide dismutase family of proteins that catalyze the oxido-reduction of ROS. Indeed, MnSOD is involved in the removal of superoxide radicals (GO: 0019430) in the mitochondrial matrix. MnSOD appears to be down-regulated in AAA samples compared to non-AAA samples. Poor data described the relationship of superoxide dismutase protein familly with abdominal aortic aneurysm, even if it is well known that oxidative stress is involved in the development of AAA. A previous study on experimental AAA in a rodent model showed an association between AAA formation and an increase in MnSOD expression in the aneurismal tissue [Sinha *et al.* 2007]. In other study, activity of MnSOD was increased in human abdominal aortic aneurismal tissue compared to control aortic tissue [Dubick *et al.* 1987]. Thus, the present study shows contrary results that have to be questioned, taking into account the influence of the technical bias described previously due to DNAse I treatment of the samples. It is also possible that differences found might be explained by the fact that Dubick *et al*. [Dubick *et al.* 1987] performed the study directly on aneurysmal tissue and macrophages studied in the present work came from circulating monocytes that were differentiated *in vitro*.

3.2. Smooth muscle cell proteomic analysis

3.2.1. Introduction

The main structural cells in the aortic wall are smooth muscle cells. They play an important role in maintaining aortic wall shape against forces, like pressure during pulse propagation, through complex contraction and relaxation mechanisms which involve cytoskeleton reorganization. They also play a key role in the regeneration of ECM since they are the only cells that able to synthesize collagen and elastin. AAA is characterized by an important depletion of SMC within the media, which contributes to increase aortic wall degradation and subsequent enlargement of the vessel diameter.

For each patient recruited in CORONA clinical protocol, smooth muscle cells were isolated from a residual segment of internal mammary artery used for the bypass, and cultured *in vitro.* Protein extracts of cultured smooth muscle cells from AAA and non-AAA patients were analyzed by 2D-DIGE.

3.2.2. Materials and methods

3.2.2.1. Isolation and culture of human aortic smooth muscle cells

For a period of 4 years (2002-2006), 24 primary cultures of aortic smooth muscle cells (ASMC) were prepared as previously described [Dupont *et al.* 2005], according to a technique patented by Dr. J. B. Michel (INSERM Patent no. 00.09055) [Battle *et al.* 1994]. Briefly, ASMC were isolated from a residual segment of human internal mammary arteries obtained from patients undergoing coronary artery bypass grafting. First, the media was stripped from the underlying adventitia and then finely minced and digested for 45 min at 37ºC in 5 mL of HAM-F10 (Gibco BRL, Grand Island, NY, US) containing 3 mg of collagenase (type I, 235 U/mg; Gibco BRL), 7 mg of elastase (3.73 U/mg; Worthington, Lakewood, NJ, USA) and 5 mg of soybean trypsin inhibitor (Sigma, St. Louis, MO, USA). Then, the enzymatic activity was stopped by adding 30% fetal calf serum (FCS) (Bio-West, Miami, Florida, USA). Cultures of ASMC were grown separately in HAM-F10 medium supplemented with 30% FCS, 10% human serum (Bio-West), 100 units/mL penicillin, 100 mg/mL steptomycin, 2.5 U/mL fungizone, 20mmol/L HEPES buffer (Gibco BRL), 2 mg/mL insulin (Roche Diagnostics, Mannheim, Germany), and 1% non essential amino acid solution (Gibco BRL). Medium was changed every 3 days. Cells were trypsinized at confluence and reseeded at a 1/2 ratio. Confluent cells at passage 2 were washed three times with Hank's Balanced Salt Solution buffer and then incubated for 24 h in serum-free culture medium before protein extraction was performed.

In 2008, commercialized human aortic smooth muscle cells (Cat. No.: C-12532, PromoCell, Germany) were cultured in the same conditions as described above. Cells were trypsinized at confluence and reseeded at an estimated concentration of 0.4 million cells per dish. Proteins were extracted from confluence cells at passage 9. This smooth muscle cell sample is referred as standard during following sections.

3.2.2.2. Extraction of intracellular proteins from human ASMCs

ASMCs were washed three times with 5 mL of 25 mM Tris, pH 7.4, and scraped into 75 µL of buffer containing 50 mM Tris pH 8.6, 10 mM EDTA, 65 mM DTT, proteinase-inhibitor cocktail (1 tablet for 10 mL buffer) (Complete; Roche Diagnostics, Meylan, France), 2,000 U/mL DNase I and 2.5 mg/mL RNase A (Roche Diagnostics) and incubated for 10 min at room temperature. Cells were then lysed in ice using a mixer suitable for 1.5 mL tubes and protein extracts were immediately stored at -20°C.

3.2.2.3. Protein labeling

The 2-D Clean-up kit (GE Healthcare) was used to clean up 50 mg of ASMC protein extracts for DTT elimination. Protein pellets were then resuspended in 20 µL of lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 30 mM Tris, pH 8) until the protein concentration was determined with the

Bio-Rad RCDC Protein Assay (Bio-Rad,UK) and BSA as the protein standard. DTT-cleaned samples were reduced and labeled with DIGE saturation dyes as described previously [Dupont *et al.* 2008]. Briefly, the procedure for the analytical gels consisted in adjusting 5 µg of every cleaned sample to 9 µL with lysis buffer, reducing it by incubation with 1 µL of 2 mM Tris (2-carboxyethyl) phosphine at 37°C for 1 h, adding 2 µL of 2 mM cyanine (Cy3 or Cy5) and incubating for 30 min at 37°C. The reaction was stopped by the addition of 12 µL of buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 130 mM DTT and 2% v/v PharmalyteTM (GE Healthcare), broad range pH 3-10 (GE Healthcare).

The procedure for preparative gels consisted in adjusting 500 µg of every sample to 250 µL with lysis buffer, reducing it by incubation with 10 µL of 20 mM Tris (2-carboxyethyl) phosphine for 1 h at 37°C, adding 20 µL of 20 mM cyanine 3 (Cy3), and incubating for 30 min at 37°C. The reaction was stopped by adding 165.5 µL of the buffer used for the analytical gels.

3.2.2.4. 2D-DIGE

Before 2D gel electrophoresis, Cy3-labeled samples (ASMC proteins from the 24 grafted patients) were mixed with the corresponding Cy5-labeled samples (24 replicates of the reference ASMC culture proteins). For the isoelectrofocusing steps, IPG strip (24 cm, linear gradient pH 3-10) were rehydrated with 450 µL of labeled sample mixture in buffer containing 7 M urea, 2 M thiourea and 4% w/v CHAPS in a Protean IEF cell system (Biorad) for 24 h without applying any current. After rehydration, IEF was performed at 300 V for 3 h, and then at a gradient to 1000 V for 6 h, at a gradient to 8000 V for 3 h and finally at 8000 V for 3 h. After IEF, the IPG strips were incubated for 10 min at room temperature with equilibration buffer containing 6 M urea, 0.1 mM Tris-HCl pH 8, 30% v/v glycerol and 2% w/v SDS, applied to the top of a 12.5% isocratic Laemmli gels, sealed with low melting temperature agarose (GE Healthcare). SDS-PAGE was run in an Ettan-Daltsix system (GE Healthcare)at 20°C and at a constant voltage of 70 V overnight followed by 300 V until the bromophenol front reached the bottom of the gel. All electrophoresis procedures (labeling, first and second dimension) were performed in the dark.

3.2.2.5. Image acquisition and bioinformatic analysis

Gels cast between two low-fluorescence glass plates were scanned with an Ettan DIGE Imager scanner (GE Healthcare) at excitation/emission wavelengths of 532 nm/580 nm for Cy3 and 633 nm/670 nm for Cy5 to yield images with a pixel size of 100 µm.

Image analysis was performed with Progenesis SameSpots v3.0 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) equipped with a specific module for the analysis of gels in which several staining were applied. Briefly, images had to be classified by pairs according to how

gels were performed, i.e. the image of each SMC protein sample together with the corresponding image of the standard sample composed a gel. Then, after an overall reference image was chosen among the images of corresponding to the standard sample, the other 2D-gel images were automatically aligned in two steps before manual verification. First, each image of the standard sample was aligned to the overall reference image, and second, the image of each SMC samples was aligned to the standard sample included in the same gel. Spot volumes were then calculated and normalized in each image. In the next step, the appropriate gels were selected for each group. The differences in protein spots were then analyzed. Spots were considered to have significantly different normalized spot volumes if the fold change was greater than 2 and the corresponding p value (one-way ANOVA analysis) was significant. The last step applied multivariate statistics to the selected spots by calculating q values (for the false discovery rate) and power. Principal component analysis was also used to determine whether the data contained any outliers.

3.2.2.6. In gel tryptic digestion and peptide extraction

Spots corresponding to proteins differentially expressed between the different groups of samples were manually excised from a preparative gel. Then, digestion was performed as previously described [Acosta-Martin *et al.* 2009]. Briefly, before digestion, spots were washed for 15 min with 100 µL of 50 mM ammonium bicarbonate solution and then twice for 15 min with 50 mM ammonium bicarbonate/50% ACN. They were then dried after adding 100 µL of ACN for 10 min. After discarding the supernatant, tubes were left open for 10 min to complete solvent evaporation. They were then rehydrated with 12 µL of a solution containing 0.025% ProteasMAX^{TM} Surfactant, Trypsin Enhancer (Promega) in 50 mM ammonium bicarbonate and 3 µL of 40 µg/mL Trypsin Gold (Promega) in 50 mM acetic acid. After overnight digestion at 37°C, peptide extraction was carried out in two steps according to the manufacturer's protocol for ProteasMAX^{TM} Surfactant, Trypsin Enhancer. First, after adding 10 µL 0.01% Surfactant solution and shaking for 10 min, the supernatant was transferred into a new tube and 20 µL of 0.01% Surfactant, 1% TFA solution were added; after the tube was shaken for 10 min, the supernatant was transferred with the first peptide extraction. Peptides were then purified, desalted with ZipTip C18 tips (Millipore Bedford, MA, USA) according to the manufacturer's protocol, and eluted with 3 µL of 0.1% TFA/50% ACN.

3.2.2.7. MALDI MS and protein identification

For acquisition of the mass spectra of the extracted and desalted peptides, we mixed 0.5 µL of the peptide solution with 0.5 µL of matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid dissolved in 0.1% TFA / 50% ACN) on the MALDI-TOF MS target. External calibration was performed with a peptide mixture resulting from the tryptic digest of BSA (0.5 µg/mL). MALDI-TOF MS was then

performed with a Voyager DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) equipped with a 337.1 nm nitrogen laser and a delayed extraction facility (125 msec). All spectra were acquired in a positive ion reflectron mode under 20 kV voltage, 61% grid. Typically, 300 laser shots were recorded per sample. The mass spectra were then calibrated using DataExplorer software (PerSeptive Biosystems, Framingham, MA, USA) before protein identification by peptide mass fingerprinting, which was conducted by running the MASCOT web searcher (http://www.matrixscience.com/, Matrix Science, UK) against the NCBInr 20080718 (6833826 sequences; 2363426297 residues) with the following parameters: Fixed modifications: CyDye-Cy3 (C); Variable modifications: Oxidation (M); Peptide Mass Tolerance: ± 50 ppm; Peptide Charge State: 1+; Max Missed Cleavages: 1.

When proteins could not be identified by peptide mass fingerprint, tandem mass spectrometry was applied at Centre d'Analyse Protéomique de l'Artois (Université d'Artois-Faculté Jean Perrin, Lens, France) as follows: The MALDI target plate (AnchorChip™, Bruker Daltonics, Bremen, Germany) was covered with a-cyanohydroxycyanimic acid (CHCA) matrix (0.3 mg/ml in acetone:ethanol, 3:6 (v/v)). Peptide mixture was directly applied onto the CHCA matrix thinlayer. The m/z measurements were performed in automatic mode using FlexControl™ 2.2 software on an Ultraflex™ TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) and in the reflectron mode for MALDI-TOF peptide mass fingerprint or LIFT mode for MALDI-TOF/TOF peptide fragmentation fingerprint. External calibration was performed using the peptide calibration standard kit (Bruker Daltonics, Bremen, Germany). Peak lists were generated from MS and MS/MS spectra using FlexAnalysis™ 2.4 software (Bruker Daltonics, Bremen, Germany). Database searches, through Mascot (Matrix Science Ltd, London, UK), were performed via ProteinScape 1.3 (Bruker Daltonics, Bremen, Germany), using the following parameters: Database: SwissProt Release 57.3; Taxonomy: Human; Fixed modifications: CyDye-Cy3 (C); Variable modifications: Oxidation (M); Peptide Mass Tolerance: ± 50 ppm; MS/MS fragment tolerance: ± 0.5 Da; Peptide Charge State: 1+; Max Missed Cleavages: 1.

3.2.3. 2D-DIGE analysis of human SMC protein samples

3.2.3.1. Morphology of human SMC

Twenty-four SMC cultures, coming from 6 AAA patients and their corresponding matched 18 non-AAA patients, were analyzed by 2D-DIGE. Figure 1 presents representative human SMC cultures of patients recruited in CORONA clinical protocol, as well as the commercial SMC culture referred as standard. All SMC cultures from patients showed the same morphology.

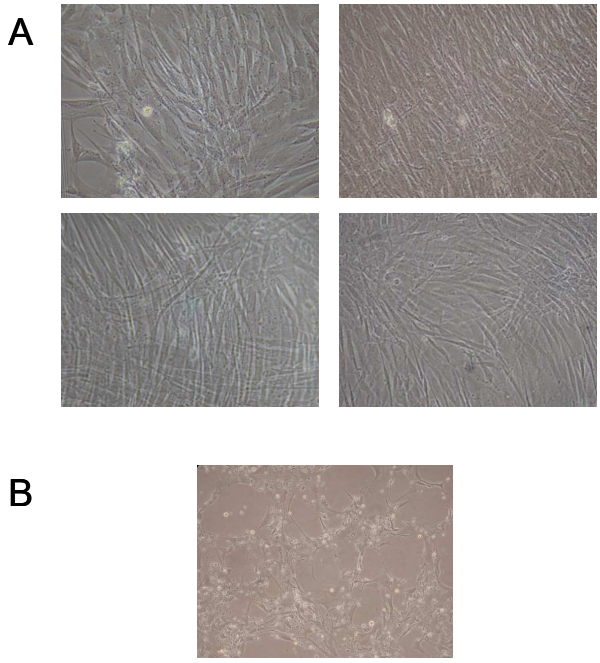


Figure 1. Morphology of human smooth muscle cells. Photographs of phase-contrast microscopy were performed on primary culture at passage 2. **A:** Examples of four primary cultures of SMCs from different patients recruited in the CORONA clinical protocol performed over a 4-year period (from 2002 to 2006). **B:** An example of the commercial primary SMC culture referred as standard.

3.2.3.2. 2D-gel pattern of human SMC

Unfortunately, in the same way as for the macrophage samples, the bioinformatic analysis of the 24 2D-gel images of SMC cultures allowed us to classify them into two groups according to the presence or absence of three specific spots (Fig. 2). Group A (4 AAA and 7 non-AAA) includes the SMC with the 3 spots present on the 2D-DIGE gels and Group B (2 AAA and 11 non-AAA), the SMC for which the 2D-DIGE gels lack these 3 spots (detailed insert in Fig. 2). These data suggested that these protein extracts from Group A SMCs appears to contain DNase I as well as Group A macrophages.

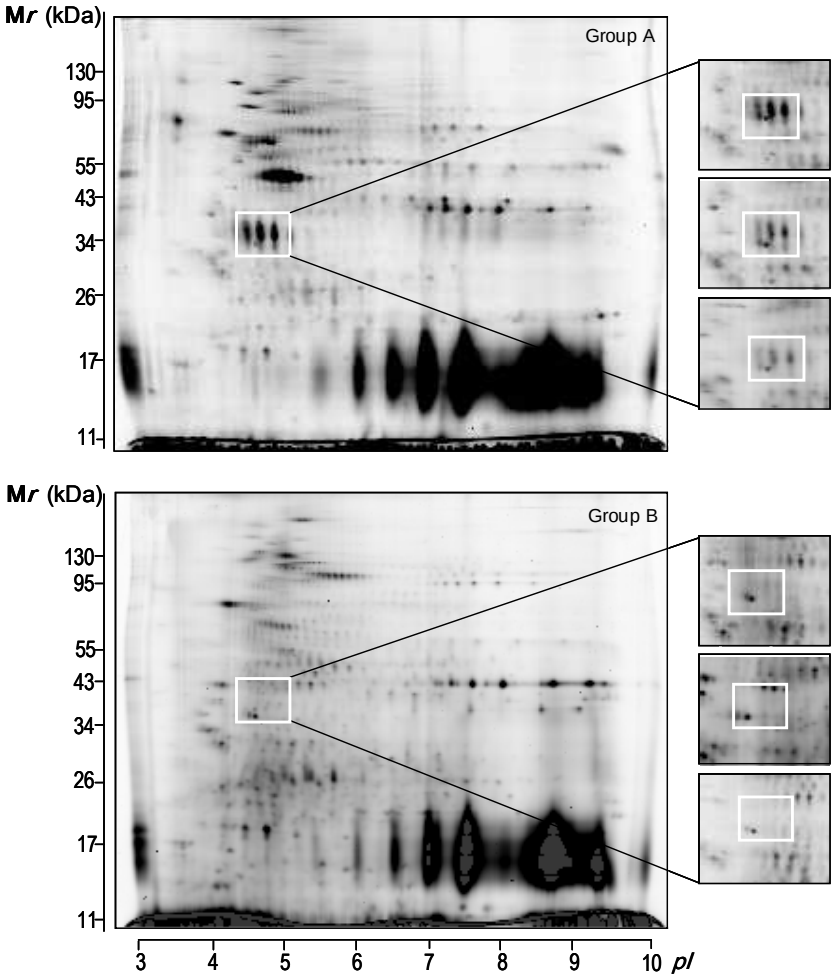


Figure 2. Representative 2D-DIGE gel analysis of intracellular proteome of human smooth muscle cells. SMC proteins (5 µg) labeled with Cy3 from cultures of 24 different SMC were analyzed. Gels were classified according to the presence (Group A) or absence (Group B) of three specific spots. Detailed insert of area discriminating between Group A or Group B is presented for three different macrophage' cultures. The positions of molecular weight (M*r*) standards are indicated on the left, and the *pI* are indicated on the bottom of the gel.

3.2.3.3. Differential protein expression between group A and group B of SMC

Because of the difference in the 2D profiles of Group A and B SMCs, despite the absence of any morphological differences (Figure 1), we performed a detailed bioinformatic analysis of the 2D gels between the two groups. DIGE labeling strategy used for the analysis of SMC protein extracts differed from the strategy used for the analysis of macrophage protein extracts. This time, a standard sample labeled with Cy5 was loaded on all gels and helped to overcome the difficulties in image alignment during bioinformatic analysis. At first glance differences in proteome profile between Group A and Group B SMC can be observed in Figure 3. Spots corresponding to the standard sample,

labeled with Cy5, colocalized with spots corresponding to Group B SMC during electrophoresis migration, but not with spots corresponding to Group A SMC, that appeared to be differently distributed along the 2D-gel. Alignment step of Group A SMC 2D-DIGE images to the standard sample in the same gel did not include any modification. We assumed that samples migrated in the same gel matrix are exposed to the same conditions and have the same behavior.

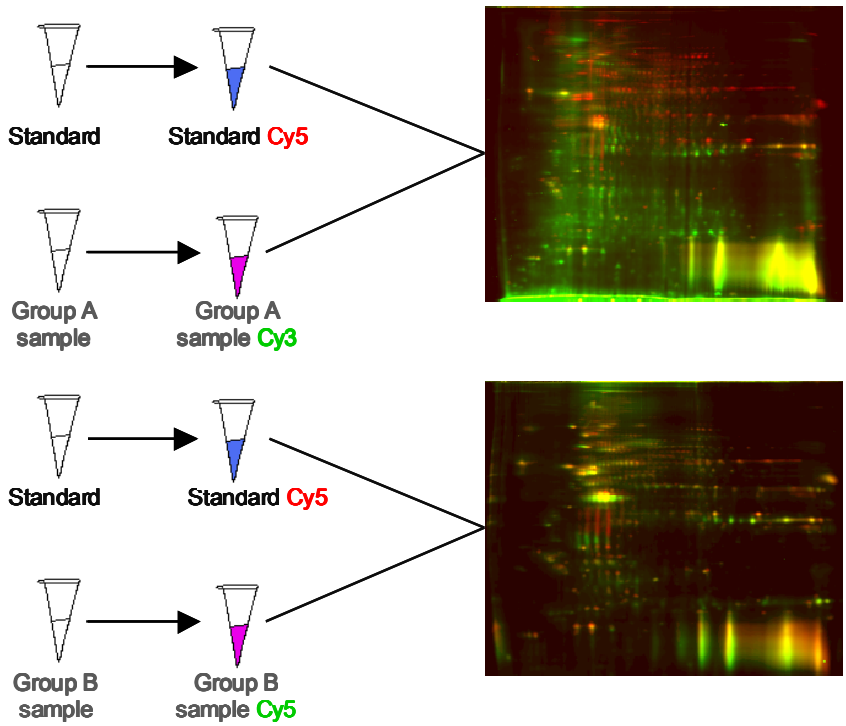


Figure 3. DIGE labeling strategy. The introduction of the standard sample in all 2D-DIGE gels facilitated alignment step during bioinformatic analysis with SameSpots software. An example of 2D-DIGE image corresponding to a gel which included a Group A SMC sample is shown in the upper part, and an example corresponding to a gel which included a Group B SMC sample is presented in the lower part. Standard sample was labeled with Cy5, in red. SMC samples were labeled with Cy3, in green. Yellow correspond to the colocalization of red and green spots within a gel.

Thanks to the labeling strategy, of 853 spots detected per gel (from 11 images of Group A and 13 of Group B), bioinformatic analysis revealed 569 polypeptide spots differentially expressed between Group A and B (p value <0.05), and of these, 408 polypeptide spots presented a fold-change equal or greater than 2. After manual validation that included requirement of a q value <0.05, 135 spots were selected as significant differentially expressed. Table 1 presents statistical values of selected spots. In all, 62 spots were up-regulated and 73 down-regulated in Group A compared to Group B (Figure 4). SameSpots software allowed us to apply principal component analysis to

determine whether the selected differential spots were reproducibly detected in all the gels from the same group (not shown).

Table 1. Statistical values of selected differentially expressed spots between Group A and Group B human SMC protein extracts

Spot	p (ANOVA)	q Value	Power	Fold Change (Group A vs. Group B)	Spot	p (ANOVA)	q Value	Power	Fold Change (Group A vs. Group B)
1	1.66E-12	8.71E-11	0.97	-28	153	3.30E-08	1.94E-07	0.63	3.4
2	2.81E-10	4.60E-09	0.85	-27	154	1.47E-09	1.67E-08	0.78	-3.4
3	8.47E-12	2.77E-10	0.95	-25	157	6.77E-07	1.88E-06	0.47	-3.4
4	8.20E-07	2.03E-06	0.46	-22	158	0.00022	0.00019	0.23	-3.4
7	1.13E-05	1.74E-05	0.34	-17	159	3.14E-10	4.85E-09	0.84	-3.3
8	1.54E-09	1.67E-08	0.78	17	160	0.00020	0.00019	0.23	-3.3
9	2.02E-12	8.88E-11	0.97	-16	161	4.63E-07	1.32E-06	0.49	-3.3
10	3.33E-16	8.78E-14	1.00	16	163	6.01E-07	1.67E-06	0.48	3.3
11	5.82E-08	3.06E-07	0.60	-14	165	3.78E-06	7.00E-06	0.39	-3.3
13	1.11E-08	8.58E-08	0.68	14	167	2.61E-07	8.88E-07	0.52	3.3
14	4.04E-08	2.37E-07	0.62	13	170	2.01E-05	2.48E-05	0.32	3.2
19	5.97E-08	3.14E-07	0.60	-12	171	1.58E-05	2.24E-05	0.33	3.2
20	2.67E-10	4.60E-09	0.85	-12	176	4.18E-06	7.75E-06	0.39	3.2
22	4.41E-07	1.32E-06	0.49	-11	178	0.00013	0.00012	0.25	3.2
24	2.17E-07	8.06E-07	0.53	-11	179	2.57E-08	1.59E-07	0.64	-3.2
25	1.49E-09	1.67E-08	0.78	-11	190	8.40E-08	3.99E-07	0.58	-3.1
27	1.31E-13	1.20E-11	0.99	11	191	8.21E-05	7.61E-05	0.26	3.1
30	1.64E-11	3.91E-10	0.93	-10	192	8.52E-06	1.45E-05	0.36	-3.1
31	3.92E-07	1.21E-06	0.50	-9.9	194	1.82E-05	2.25E-05	0.32	3.1
33	1.57E-05	2.24E-05	0.33	-9.7	196	1.36E-07	5.46E-07	0.55	-3

34	1.33E-05	2.06E-05	0.34	-9.3
35	1.06E-05	1.64E-05	0.35	-9.2
36	6.09E-11	1.13E-09	0.90	8.9
37	1.36E-13	1.20E-11	0.99	8.9
38	4.44E-08	2.47E-07	0.61	-8.8
44	1.11E-07	4.72E-07	0.56	-8.5
45	5.19E-07	1.44E-06	0.49	-8.5
46	3.88E-12	1.45E-10	0.96	-8.4
48	4.27E-07	1.32E-06	0.50	-7.9
49	1.11E-06	2.75E-06	0.45	-7.8
51	2.16E-09	2.07E-08	0.76	-7.7
52	7.02E-13	4.62E-11	0.98	7
58	5.54E-08	2.91E-07	0.60	-6.4
59	7.18E-05	6.66E-05	0.27	-6.3
60	3.02E-06	6.16E-06	0.40	-6.3
62	1.41E-11	3.71E-10	0.94	6.3
65	1.15E-08	8.70E-08	0.68	6
67	8.18E-07	2.02E-06	0.46	-5.9
69	1.05E-11	3.05E-10	0.94	-5.9
70	8.06E-05	7.48E-05	0.27	-5.7
71	1.50E-05	2.24E-05	0.33	5.7
72	1.10E-09	1.36E-08	0.79	5.7
74	3.50E-07	1.08E-06	0.51	-5.5
75	0.00027	0.00019	0.22	-5.4
76	1.69E-06	3.67E-06	0.43	-5.3
80	1.73E-09	1.82E-08	0.77	5.1
198	8.32E-05	7.72E-05	0.26	-3
200	5.71E-07	1.59E-06	0.48	-3
203	2.25E-07	8.24E-07	0.53	3
204	0.00010	9.49E-05	0.26	3
205	4.28E-05	4.22E-05	0.29	-3
207	4.23E-06	7.84E-06	0.39	2.9
212	0.00012	0.00011	0.25	-2.9
214	1.51E-05	2.24E-05	0.33	2.9
215	2.39E-07	8.24E-07	0.53	-2.9
219	1.73E-05	2.24E-05	0.33	2.9
225	9.23E-06	1.45E-05	0.35	-2.8
233	2.01E-05	2.48E-05	0.32	-2.8
235	2.91E-05	3.60E-05	0.30	-2.8
236	4.28E-06	7.94E-06	0.39	2.8
239	0.00039	0.00024	0.21	-2.7
248	1.96E-05	2.43E-05	0.32	2.7
254	9.37E-06	1.45E-05	0.35	2.6
257	2.24E-05	2.77E-05	0.31	-2.6
262	2.46E-06	5.32E-06	0.41	2.6
263	1.05E-05	1.63E-05	0.35	-2.6
264	1.82E-05	2.24E-05	0.32	-2.6
265	3.03E-05	3.75E-05	0.30	2.6
270	1.98E-05	2.45E-05	0.32	2.5
271	2.54E-06	5.50E-06	0.41	-2.5
277	6.73E-05	6.24E-05	0.27	2.5
278	3.90E-05	4.22E-05	0.29	2.5

82	1.10E-07	4.72E-07	0.57	-5.1	279	1.54E-05	2.24E-05	0.33	2.5
89	2.88E-08	1.78E-07	0.63	4.6	281	1.52E-06	3.29E-06	0.43	-2.4
92	1.17E-08	8.70E-08	0.68	-4.5	295	2.35E-05	2.90E-05	0.31	2.4
94	3.26E-05	4.03E-05	0.30	-4.5	301	0.00023	0.00019	0.23	2.4
96	2.43E-11	5.33E-10	0.92	4.5	306	0.00056	0.00035	0.20	2.4
99	1.96E-07	7.27E-07	0.54	4.4	322	0.01707	0.00528	0.11	2.3
103	6.92E-08	3.42E-07	0.59	4.3	326	0.00066	0.00041	0.19	2.2
106	2.67E-11	5.36E-10	0.92	4.2	327	3.68E-05	4.22E-05	0.29	2.2
109	1.54E-05	2.24E-05	0.33	4.2	328	0.00019	0.00018	0.23	-2.2
113	1.15E-07	4.72E-07	0.56	4	329	0.0011	0.00066	0.18	2.2
114	1.37E-05	2.12E-05	0.34	-4	340	7.76E-05	7.20E-05	0.27	2.2
116	9.42E-10	1.22E-08	0.80	3.9	343	2.91E-05	3.60E-05	0.30	2.2
120	4.27E-05	4.22E-05	0.29	-3.9	347	0.00069	0.00043	0.19	2.2
124	2.01E-09	1.99E-08	0.76	-3.8	355	1.24E-07	4.99E-07	0.56	2.2
126	7.58E-08	3.75E-07	0.58	3.8	361	1.00E-06	2.47E-06	0.45	-2.1
130	5.40E-06	1.00E-05	0.38	3.7	364	4.24E-07	1.31E-06	0.50	-2.1
131	4.26E-06	7.91E-06	0.39	3.7	365	0.00013	0.00012	0.25	2.1
132	0.00012	0.00011	0.25	-3.7	375	2.23E-06	4.82E-06	0.42	-2.1
135	7.67E-10	1.04E-08	0.81	3.6	379	0.00032	0.00020	0.22	-2.1
141	9.95E-09	8.30E-08	0.69	3.6	385	5.21E-05	4.83E-05	0.28	-2
144	1.29E-06	2.98E-06	0.44	-3.5	392	0.00051	0.00032	0.20	2
147	8.60E-08	3.99E-07	0.58	-3.5					

Fold Change is shown as a mean of normalized volume of the same spot in gels of AAA patients compared to the corresponding values of non-AAA patients.

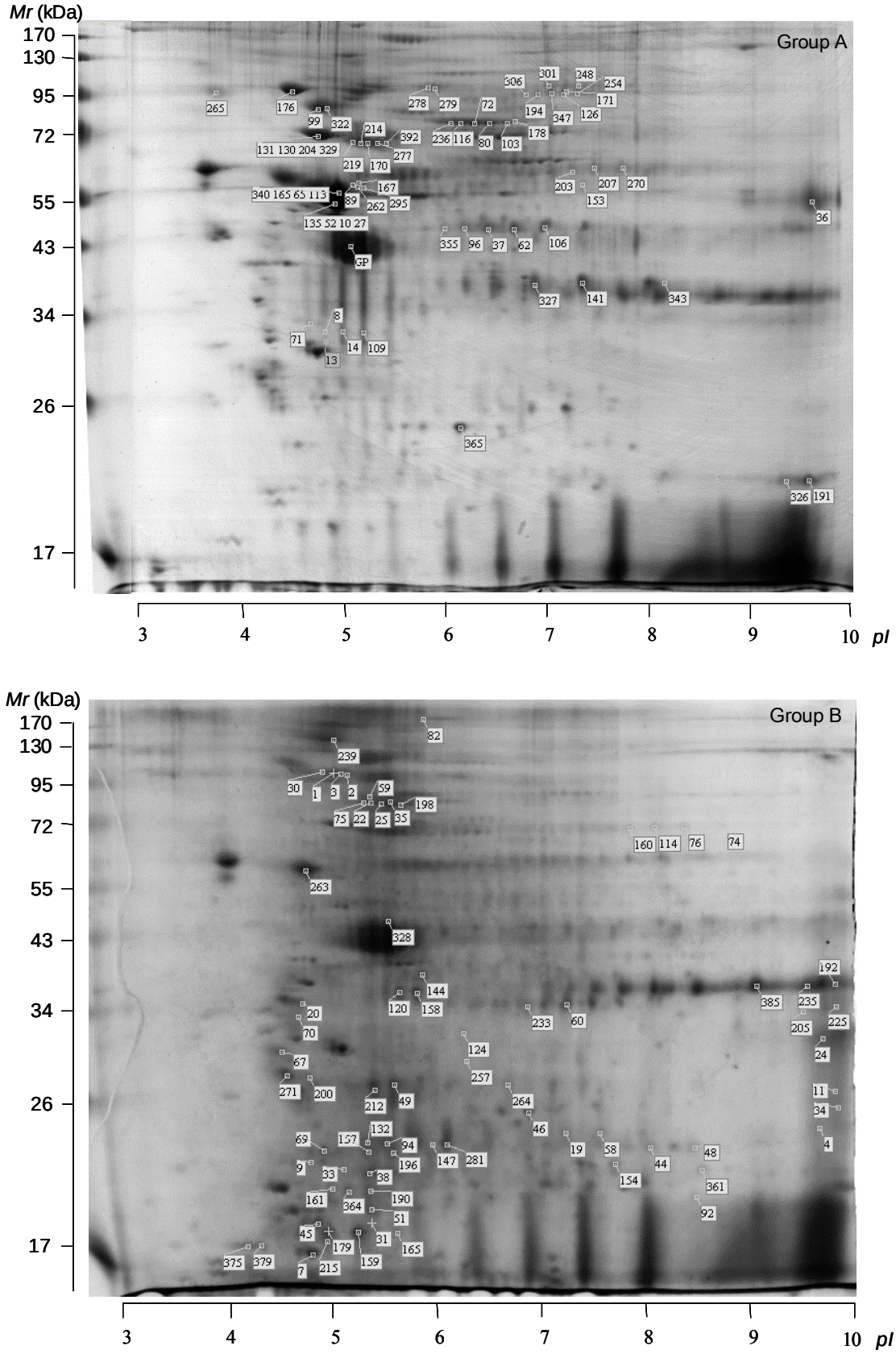


Figure 4. Selected differentially modulated spots during bioinformatic analysis. Preparative 2D-gels of human SMC protein extracts (500 µg protein) are shown. Up-regulated spots belonging to Group A are presented in

the upper part and to Group B in the lower part. The positions of molecular weight (M*r*) standards are indicated on the left, and the *pI* are indicated on the bottom of the gel.

3.2.3.4. Effect of incomplete DNAse I/RNAse A treatment on SMC patterns

Of the 135 polypeptide spots found to be differentially expressed between Group A and Group B, 31 could not be identified (spots 4, 8, 11, 14, 24, 33, 34, 38, 44, 46, 48, 58, 59, 74, 76, 92, 103, 124, 154, 165, 167, 179, 200, 215, 225, 233, 257, 264, 295, 301, and 361) due to the low intensity signal from mass spectrometry analysis or the low probability score. Unexpectedly, bovine DNAse I (UniProtKB/SwissProt accession number: P00639) was identified in 3 over-expressed and 4 under-expressed spots in group A SMC (Table 2).

Table 2. Summay of spots which protein identification corresponded to bovine DNase I

Spot number	Fold change (Group A vs. Group B)	PMF MASCOT score	matched peptides/total peptides	Sequence coverage	MS/MS analysis
8	17	44 (NS)	4/17	15	-
14	13	55 (NS)	4/13	15	-
94	-4.5	66	5/12	17.7	C MS/MS
109	4.2	62	4/9	28	-
147	-3.5	64	6/13	17.7	C MS/MS
196*	-3	60	5/15	17.7	C MS/MS
281*	-2.4	NS			K.LLDYLNQDDPNTYHYVVSEPLGR.N (110)

PMF = peptide mass fingerprint. NS = non-significant. C MS/MS = Identification confirmed by MS/MS analysis. When bovine DNase I was identified only by MALDI-TOF MS/MS analysis, the sequence of the matched peptide as well as the corresponding significant based mowse score are shown. * Peptide mass fingerprint search, or MS/MS analysis gave more than one protein with a significant probability based mowse score.

In Group A SMC, DNAse I was significantly identified in spot 109, one of the three spots used for the profile classification. For the other two spots (spots 8 and 14), the MS MASCOT search did not get any significant results but the first hit was also for bovine DNAse I. These results correlated with the results obtained in human macrophage proteomic analysis [Acosta-Martin *et al.* 2009]. Furthermore, in Group B, over-expressed spots 94, 147, 196, and 281 also appeared to correspond to

bovine DNAse I. This fact suggested that during protein profile modification by complete DNAse I treatment in group B SMC, DNAse I also underwent post-translational modifications or cleavages.

Table 3 reports the identity of the 43 different proteins corresponding to the differentially expressed polypeptide spots between Group A and Group B SMC. Interestingly, ten proteins (heat shock protein 70kDa, α-enolase, glyceraldehyde-3-phosphate dehydrogenase, annexin A2, vimentin, actin cytoplasmic 1, actin cytoplasmic 2, α-actinin 1, elongation factor 2, and fibronectin) were represented by at least five spots. Identified proteins were classified according to their biological significance: cell redox homeostasis, protein kinase cascade, stress response, protein folding, metabolic process, carbohydrate metabolic process, glycolysis, signal transduction, positive regulation of cell adhesion, skeletal system development, cell motion, negative regulation of cell motion, microtubule-based movement, translational elongation, muscle development, muscular filament sliding, regulation of cell shape, actin filament reorganization, actin cytoskeleton reorganization, anti-apoptosis, apoptosis, induction of apoptosis, and regulation of apoptosis.

Table 3. Detailed list of proteins with statistical differential expression between Group A and B SMCs

Spots number	Accession number	Protein name	Protein function	Subcellular localization	Fold change (group A *vs.* group B)	PMF MASCOT score	Matched peptides / Total peptides	Protein coverage (%)	MS/MS analysis
GO:0045454 Cell redox homeostasis									
263	P07237	Protein disulfide isomerase	Catalyzes the formation, breakage and rearrangement of disulfide bonds	Endoplasmic reticulum	-2.6	107	10/25	27.4	C MS/MS
GO:0007243 Protein kinase cascade									
265	P14314	Glucosidase 2 subunit β	Glycan metabolism	Endoplasmic reticulum	2.6	78	7/30	16	-
GO:0006950 Stress response									
67	B0QZ61	Heat shock protein 70kDa (Glucose regulated protein. 78kDa)	Molecular chaperone	Endoplasmic reticulum	-5.9	NS	-	-	K.DNHLLGTFDLTGIPPAPR.G (142)
130					3.7	69	6/19	16	-
131					3.7	78	7/18	15	-
204					3	133	12/21	21	-
329					2.2	122	9/12	19	-
GO:0006457 Protein folding									
170	P11142	Heat shock protein 71 kDa	Molecular chaperone	Cell surface	3.2	120	14/33	29	-
214					2.9	70	9/22	17	-
219					2.9	69	7/23	20	-
277					2.5	115	14/34	28	-
99	P08238	Heat shock protein HSP 90-β	Molecular chaperone	Cytoplasm	4.4	94	12/40	23	-
70	Q15084	Protein disulfide-isomerase A6	Catalyzes the rearrangement of	Endoplasmic reticulum	-5.7	113	7/14	34	-

			disulfide bonds						
GO:0008152 Metabolic process									
114	P29401	Transketolase	Transketolase activity	Cytosol	-4	80	7/31	31	-
160					-3.3	124	11/35	43	-
GO:0005975 Carbohydrates metabolic process									
278	Q14697	Neutral α-glucosidase AB	Glucan 1.3-α-glucosidase activity	Endoplasmic reticulum	2.5	198	21/40	29	-
279					2.5	161	14/40	22	-
GO:0006096 Glycolysis									
37	P06733	α-Enolase	Multifunctional enzyme	Plasmic membrane	8.9	95	13/34	36	-
62					6.3	86	14/40	36	-
96					4.5	96	13/40	33	-
106					4.2	133	14/34	41	-
355*					2.2	134	11/40	31	-
203*	P14618	Pyruvate kinase isozymes M1/M2	Glycolytic enzyme	Cytosol	3	191	13/40	25	-
207					2.9	132	16/40	33	-
270					2.5	149	17/40	36	-
19	P04406	Glyceraldehyde-3-phosphate dehydrogenase	Membrane traficking	Cytoplasm	-12	NS	-	-	K.LISWYDNEFGYSNR.V (28)
60					-6.3	73	5/13	26	-
192					-3.1	75	7/23	34	-
235					-2.8	75	7/23	34	-
385					-2	85	9/17	32	-
GO:0007165 Signal transduction									
153	Q01518	Adenylyl cyclase-associated protein 1	Regulates filament dynamics	Plasmic membrane	3.4	103	13/40	36	-
GO:0045785 Positive regulation of cell adhesion									

322	P21980	Protein-glutamine γ-glutamyltransferase 2	Catalyzes conjugation of polyamines to protein	Plasmic membrane	2.3	183	20/40	33	-
GO:0001501 Skeletal system development									
31	P07355	Annexin A2	Calcium regulated membrane binding protein	Basement membrane	-9.9	73	8/40	25	-
51					-7.7	78	8/39	32	-
141					3.5	82	8/25	25	-
327					2.2	92	10/37	32	-
343					2.2	127	12/40	42	-
GO:0006928 Cell motion									
7	P08670	Vimentin	Class-III intermediate filaments	Cytoplasm	17	136	13/21	24.1	C MS/MS
10					16	146	17/41	41.5	C MS/MS
27					11	78	8/40	37	-
36					8.9	63	9/30	22.8	C MS/MS
45					-8.5	NS	-	-	R.ISLPLPNFSSLNLR.E (93)
52					7	218	21/61	54.8	C MS/MS
65					6	97	11/40	32	-
113					4	106	12/40	31	-
161					-3.3	70	6/35	28	C MS/MS
163					3.3	93	9/19	28	-
236*					2.8	101	9/11	19.1	C MS/MS
340*					2.2	204	28/65	53.8	C MS/MS
135	P07437	Tubulin-β-chain	Major constituent of microtubules	Microtubule	3.6	74	9/15	19	-
340*					2.2	88	15/64	43	C MS/MS
72	Q05682	Caldesmon	Regulates the interaction between actin and calmodulin.	Cytoskeleton	5.7	74	6/12	12	-
80					5.1	59	11/33	13	C MS/MS

116			myosin and tropomyosin		3.9	95	16/41	17.8	C MS/MS
178					3.2	60	11/15	12.5	-
49*	P60709	Actin, cytoplasmic 1	Involved in various types of cell mobility	Cytoskeleton cytoplasm	-7.8	72	5/15	25	-
144*					-3.5	110	10/27	40	-
212*					-2.9	103	8/26	41	-
328*					-2.2	92	10/40	31	-
355*					2.2	78	7/40	26	-
49*	P63261	Actin, cytoplasmic 2	Involved in various types of cell mobility	Cytoskeleton cytoplasm	-7.8	71	5/15	25	-
120					-3.9	72	6/30	27	-
144*					-3.5	109	10/27	40	-
212*					-2.9	114	8/26	41	-
328*					-2.2	89	10/40	31	-
355*					2.2	76	7/40	26	-
GO:0051271 Negative regulation of cell motion									
22	P12814	α-Actinin 1	Bonds actin to different intracellular structures	Cytoplasm	-11	105	15/40	21	-
25					-11	100	15/40	20	-
35					-9.2	83	14/40	17	-
75					-5.4	105	15/39	22	-
158					-3.4	116	15/40	21	-
198					-3	76	11/29	15	-
GO:0007018 Microtubule-based movement									
89*	Q71U36	Tubulin α-1A chain	Major constituent of microtubules	Microtubule	4.6	69	8/30	27	-
236*					2.8	NS	-	-	R.AVFVDLEPTVIDEVR.T (39)
262*					2.6	71	7/28	22	-
340*					2.2	77	11/64	32.7	-

89*	P68363	Tubulin α-1B chain	Major constituent of microtubules	Microtubule	4.6	69	8/30	27	-
262*	Q9BQE3 P68366	Tubulin α-1C chain Tubulin α-4A chain			2.6	71	7/28	22	-
GO:0006414 Translational elongation									
126	P13639	Elongation factor 2	Promotes the GTP-dependent translocation	Cytoplasm	3.8	96	11/23	16	-
171					3.2	120	17/40	22	-
194					3.1	80	21/40	28	-
248					2.7	96	14/39	16.6	-
254					2.6	100	5/20	18	-
306					2.4	140	19/40	25	-
347					2.2	79	11/29	17	-
GO:0007517 Muscle development									
191	Q01995	Transgelin	Implicated in cell contractile properties	Cytoplasm	3.1	78	10/40	49	-
326				Cytoplasm	2.2	75	11/40	39	-
GO:0030049 Muscular filament sliding									
375	P60660	Light-myosin polypeptide 6	Myosin regulation	Myosin complex	-2.1	76	5/12	30	C MS/MS
379					-2.1	68	6/41	42	-
GO:0008360 Regulation of cell shape									
1*	P02751	Fibronectin	Extracellular matrix structural constituent	RE-Golgi intermediate compartment	-28	212	16/40	14	-
3*					-25	172	14/40	13	-
30					-10	80	11/25	12	-
82					-5.1	122	16/40	16	-
239					-2.7	166	17/24	17	-
GO:0007015 Actin filament reorganization									
203*	Q16658	Fascin	Actin binding protein	Cytoplasm	3	191	9/40	22	-

392*	P13797	Plastin-3	Actin binding protein	Cytoplasm	2	126	9/35	19	-
GO:0031532 Actin cytoskeleton reorganization									
1*	P21333	Filamin-A	Links actin filament to membrane glycoproteins	Actin cytoskeleton	-28	212	17/40	12	-
2					-27	87	15/29	8.8	C MS/MS
3*					-25	172	17/40	12	-
9*	P11233	Ras-related protein Ral-A	Belongs to the small GTPase superfamily	Plasmic membrane	-16	72	8/40	50	-
190*					-3.1	NS	-	-	K.INVNEIFYDLVR.Q (68)
364*					-2.1	NS	-	-	K.INVNEIFYDLVR.Q (60)
GO:0006916 Anti apoptosis									
176	P14625	Endoplasmin	Molecular chaperone	Endoplasmic reticulum	3.2	135	20/40	24	-
365	P04792	Heat shock protein β-1	Involved in stress resistance and actin reorganization	Cytoplasm	2.1	123	10/47	46.3	C MS/MS
392*	P08107	Heat shock protein 70 kDa 1	Molecular chaperone	Endoplasmic reticulum	2	126	6/35	21	-
13	P08758	Annexin A5	Involved in coagulation cascade	Cytoplasm	14	85	9/40	37	-
71					5.7	86	8/40	35	-
69	P09211	Glutathione S-transferase P	Glutathione transferase activity	Cytoplasm	-5.9	NS	-	-	K.FQDGDLTLYQSNTILR.H (57)
132					-3.7	68	5/22	45	-
157					-3.4	68	5/32	45	-
196*					-3	69	5/15	39	C MS/MS
281*	P07996	Thrombospondin-1	Mediates cell-to-cell and cell-to-matrix interactions	Extracellular matrix	-2.4	69	6/36	41	-
GO:0006915 Apoptosis									
205	P21796	Voltage-dependent anion-selective channel protein 1	Catalyzes transmembrane anion transfer	Plasmic membrane	-3	125	12/40	51	-

271	P62258	14-3-3 Protein epsilon	Implicated in signaling pathways	Cytoplasm	-2.5	71	9/31	31.5	C MS/MS
GO:0006917 Induction of apoptosis									
190*	P63241	Eukaryotic translation initation factor 5A-1	Involved in mRNA turnover	Cytoplasm	-3.1	NS	-	-	R.NDFQLIGIQDGYLSLLQDSGEVR.E (66)
364*				Endoplasmic reticulum	-2.1	NS	-	-	R.NDFQLIGIQDGYLSLLQDSGEVR.E (146)
GO:0042981 Regulation of apoptosis									
159	P09382	Galectin-1	Regulates apoptosis. proliferation and cell differentiation	Cytoplasm	-3.3	111	8/43	50.7	C MS/MS
Non classified									
20	Q07065	Cytoskeleton-associated protein 4	/	Membrane	-12	74	19/36	23	-

Monoisotopic peptides masses were searched for NCBI protein database for peptide mass fingerprint and UniProtKB/SwissProt database for MS/MS searches using MASCOT search engine (http://www.matrixscience.com/). Assignments were made according to UniProtKB/SwissProt Release 15.1 / 57.1 of April 14, 2009 (462,764 entries) PMF = peptide mass fingerprint. NS = non-significant. C MS/MS = Identification confirmed by MS/MS analysis. When proteins were identified only by MALDI-TOF MS/MS analysis, the sequence of the matched peptide as well as the corresponding significant based mowse score are shown. * peptide mass fingerprint search or MS/MS analysis gave more than one protein with a significant probability based mowse score, all possible results are shown in the table.

More than one half of significantly differential expressed proteins between Groups A and B can be classified into two groups of protein families. The first group comprised 10 proteins involved in apoptosis (endoplasmin, heat shock protein β-1, heat shock protein 70 kDa 1, annexin A5, glutathione S-transferase P, thrombospondin-1, voltage-dependent anion-selective channel protein 1, 14-3-3 protein epsilon, eukaryotic translation initiation factor A5-1 and galectin-1). Six of these proteins were down-regulated (glutathione S-transferase P, thrombospondin-1, voltage-dependent anion-selective channel protein 1, 14-3-3 protein epsilon, eukaryotic translation initiation factor A5-1, and galectin-1) and four up-regulated (endoplasmin, heat shock protein β-1, heat shock protein 70 kDa 1, and annexin A5) in Group A compared with Group B.

The second group, comprised 18 proteins involved in cell motion and actin reorganization: vimentin, tubulin-β-chain, caldesmon, actin cytoplasmic 1, actin cytoplasmic 2, α-actinin, tubulin α-1A chain, tubulin α-1B chain, tubulin α-1C chain, tubulin α-4A chain, elongation factor 2, transgledin, light-myosin polypeptide 6, fibronectin, fascin, plastin-3, filamin A, and ras-related protein RaI-A. Five of these proteins were down-regulated (α-actinin, light-myosin polypeptide 6, fibronectin, filamin A, and ras-related protein RaI-A) and ten up-regulated (tubulin-β-chain, caldesmon, tubulin α-1A chain, tubulin α-1B chain, tubulin α-1C chain, tubulin α-4A chain, elongation factor 2, transgledin, fascin, and plastin-3) in Group A compared with Group B. Three proteins (vimentin, actin cytoplasmic 1, and actin cytoplasmic 2) appeared in spots that were both up and down-regulated in Group A compared to Group B, a situation that suggests a possible differential post-translational modification or protein cleavages.

These results confirmed previous results found during the 2D-DIGE proteomic analysis of macrophage protein extracts, in which treatment with DNase I clearly explained why the main differences in protein expression profiles between Group A and Group B were for proteins involved in actin reorganization and cell motion. Moreover, the specific complexity of SMC cytoskeleton for relaxation and contraction processes made that a higher number of proteins involved in actin reorganization and cell motion were affected by the bias in this cell type. Besides confirmation of previous results, 2D-DIGE analysis of DNase I treated SMC protein extracts revealed another major group of affected proteins, i.e. proteins involved in apoptosis and its regulation. Indeed, DNase I has been described to play an important role in apoptosis [Mannherz *et al.* 1995], particularly in the final phases of DNA cleavage. Thus, sudden increased amounts of DNase I may produce a deregulation of expression of proteins involved in apoptotic processes. Interestingly, the interaction between actin and DNase I was proposed to have a function in protection of cells from premature cell death [Eulitz *et al.* 2007], so we could hypothesize that most of differential protein expressions found in Group B compared to Group A SMC are a consequence of the complex interaction between DNase I and actin,

and collateral effects in other proteins involved in both cytosketelon reorganization and apoptosis. During proteomic analysis of macrophage protein extracts, only two proteins were found to be involved in apoptosis (glutathione-S-transferase P and 14-3-3 protein zeta/delta). The reason why a greater number of proteins involved in apoptosis and its regulation were found in SMC samples compared to macrophages samples is not known. Probably, DIGE labeling strategy in SMC protein extracts, that allowed for a better alignment between Group A and Group B proteome profiles, gave rise to the detection of differentially expressed spots that could not be noticed during the bioinformatic analysis of 2D-DIGE gels of macrophage protein extracts. Actually, 104 spots were found to be differentially expressed in the analysis of macrophage samples while 135 spots were found in the analysis of SMC samples, corresponding to 31 and 43 identified proteins, respectively.

3.2.3.5. Differential protein expression between AAA and non-AAA SMC samples

Regarding disease status classification of the samples, proteomic profile of 2D-DIGE gels of AAA and non-AAA samples were compared following two different strategies: with, and without consideration of the classification in Group A and Group B proteomic profile.

When proteomic profile classification was not taken into account, corresponding images of 2D-DIGE gels of SMC protein extracts of 6 AAA patients were compared to corresponding images of 18 non-AAA patients. When gels were analyzed within group A of proteome profiles, corresponding images of 2D-DIGE gels of SMC protein extracts of 4 AAA patients were compared to corresponding images of 7 non-AAA patients. When gels were analyzed within group B of proteome profiles, corresponding images of 2D-DIGE gels of SMC protein extracts of 2 AAA patients were compared to corresponding images of 11 non-AAA patients. For all three comparisons between 2D-DIGE gels of AAA and non-AAA SMC samples, bioinformatic analysis revealed, after manual validation, that none of the 853 spots detected *per* image was significantly differentially expressed.

3.2.4. General conclusion on cell sample analysis

The aim of the differential proteomic analysis on macrophage and SMC protein extracts from patients recruited in CORONA clinical protocol was the comparison of protein expression between samples from AAA patients and non-AAA patients. The use of saturation DIGE, a sensible technique in the case of scarce samples, together with SameSpots software, is a powerful tool that have allowed the identification of 2 different protein profiles in both cell types independently on the disease status of the patient. These two different proteome profiles are the result of a technical bias during the application of DNAse I treatment on the samples for the elimination of nucleic acids. The 2D-DIGE study of the protein expression profiles of macrophages and SMC of patients recruited in CORONA clinical protocol showed that DNase I treatment produced changes in proteome profiles of these cell types. The proteins mainly affected by this treatment were those involved in cell motion, actin

cytoskeleton reorganization, and apoptosis. Indeed, DNase I has been reported to be involved in cell apoptosis, and in actin cytoskeleton regulation because of its high affinity for actin [Mannherz *et al.* 1980; Morrison *et al.* 2007]. Due to the presence of this technical bias, comparison between AAA and non-AAA cell samples did not get any concluding result. The use of DNase I for the removal of nucleic acids from protein extracts of cell samples must be avoided. We strongly recommend the use of alternative techniques, such as sonication or precipitation, to remove nucleic acids from protein samples. Another potential recently proposed technique is a two-phase extraction of DNA in chloroform/phenol/isoamyl alcohol to remove DNA from E. coli extracts [Antonioli *et al.* 2009].

4. Analysis of plasma samples

4.1. Introduction

Plasma samples represented the most challenging samples to be analyzed in this project, not only because of the inherent difficulties of the proteomic analysis of plasma samples themselves, but also because they are representative of samples used clinically for biomarker detection and diagnosis of most diseases. Thus, findings from the differential proteomic analysis stage are critical, but subsequent capabilities to easily detect differential expressed proteins in patient plasma samples are indispensable for the success of the first step of biomarker discovery.

Plasma samples were analyzed by two different quantitative proteomic strategies: by 2D-gel based differential proteomics in my home laboratory, in Lille, and by nLC-MS/MS quantitative proteomic analysis during a 3-month stay in the laboratory of Pr. David R. Goodlett, in Seattle (USA).

4.2. 2D-gel analysis of plasma samples

4.2.1. Materials and methods

4.2.1.1. Plasma sample preparation

Plasma samples of 13 AAA patients as well as 26 non-AAA patients, perfectly coupled according to the patients matching, were used for the proteomic analysis.

In a first analysis, a total volume of 100 µL of each plasma sample was depleted for albumin and IgG using the ProteoPrep Blue Albumin and IgG Depletion kit (Sigma, Saint Louis, Missouri) according to the manufacturer's protocol. After depletion, protein concentration was determined with the Bio-Rad R_CD_C Protein Assay (Bio-Rad, UK) and BSA as the protein standard.

In a second analysis, 900 µg of each plasma sample were pooled in two groups according to patient classification as AAA or non-AAA. Then, pooled plasma were depleted and quantified as described above.

4.2.1.2. Two dimensional electrophoresis and silver staining

After depletion, volume corresponding to 100 µg of each plasma protein sample was adjusted to 200 µl with IPG loading buffer (Genomic Solutions, Ann Arbor, MI) and then mixed with 200 µl of IPG rehydration buffer (Genomic Solutions, Ann Arbor, MI). For the isoelectrofocusing steps, IPG strip (18 cm, linear gradient pH 4-7) were rehydrated with 400 µL of sample mixture in a Protean IEF cell system (Biorad) for 9 h without applying any current and then for 9 h at 50 V. After rehydration, IEF was performed at 200 V for 1 h, and then at a gradient to 1,000 V for 1 h, at a gradient to 10,000 V for 6 h and finally a step and hold at 10,000 V for 4.5 h. Temperature was maintained at 20ºC for all IEF steps. After IEF, the IPG strips were equilibrated firstly by incubation for 15 min at room temperature with reducing buffer containing 0.02% w/v DTT (dithiothreitol), and secondly for 15 min at room temperature with alkylation buffer containing 0.025% w/v IAA (iodoacetamide). Equilibrated IPG strips were applied to the top of a 12.5% isocratic Laemmli gels, sealed with low melting temperature agarose (GE Healthcare). SDS-PAGE was run in an Ettan-Daltsix system (GE Healthcare) at 20°C and at a constant voltage of 70 V overnight followed by 300 V until the bromophenol front reached the bottom of the gel.

Once electrophoresis finished, gels were fixed for 2 h at room temperature in buffer containing 30% ethanol and 5% acetic acid. Fixed gels were washed four times with deionized water for 10 min. Then, gels were incubated for 1 min with sensitizing solution containing 0.02% w/v sodium thiosulfate, and washed twice with deionized water for 1 min. After sensitization, gels were incubated for 45 min with staining buffer containing 0.002% w/v silver nitrate and 0.00028% v/v formaldehyde. Gels were developed by incubation with buffer containing 0.024% w/v sodium carbonate, 0.000013% sodium thiosulfate, and 0.00028% v/v formaldehyde. When spots were correctly visualized, gels were incubated for 30 min with terminating solution containing 0.04% w/v Tris base and 0.02% v/v acetic acid. Finally gels were rinsed twice with deionized water.

For pooled plasma samples, gels were performed in six replicates.

4.2.1.3. Image acquisition and bioinformatic analysis

Silver-stained 2-D gels were digitized at 14 bits and 300 densities per inch (dpi) resolution using an Imagescanner® scanner (GE Healthcare). Image analysis was performed with Progenesis SameSpots v2.0 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Briefly, after a reference image was chosen, the other 2D-gel images were automatically aligned before manual verification. Spot volumes were then calculated and normalized in each image. In the next step, the appropriate gels were selected for each group. The differences in protein spots were then analyzed. Spots were considered to have significantly different normalized spot volumes if the fold change was greater

than 1.5 and the corresponding p value (one-way ANOVA analysis) was significant. The last step applied multivariate statistics to the selected spots by calculating q values (for the false discovery rate) and power. Principal component analysis was also used to determine whether the data contained any outliers.

4.2.2. Results on silver-stained 2D-gel analysis of plasma samples

4.2.2.1. Differential protein expression between AAA and non-AAA plasma samples by individual sample analysis

Silver-stained 2D-gels (Figure 1) of depleted plasma samples of 8 AAA and 16 non-AAA were performed and used for the bioinformatic analysis. After manual validation, bioinformatic analysis revealed that none of the 882 spots detected *per* image was significantly differentially expressed between both groups of plasma samples.

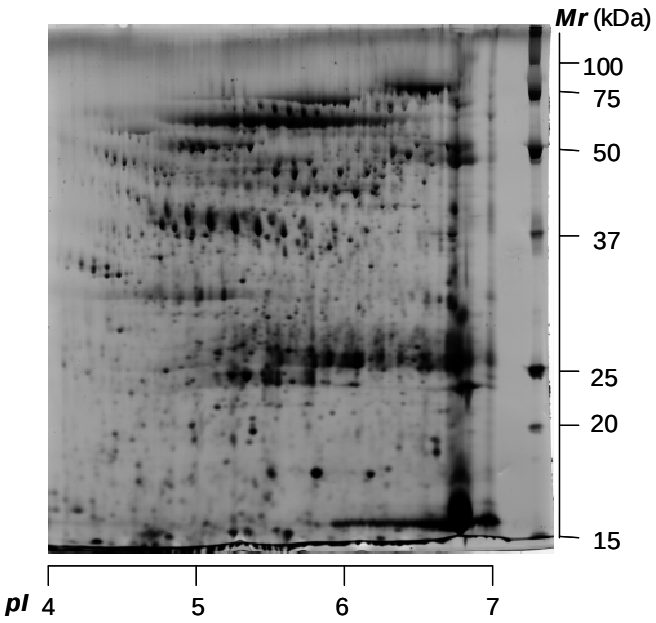


Figure 1. Representative silver-stained 2D-gel of a depleted plasma sample. A hundred µg of each depleted plasma proteins was used to perform two-dimensional electrophoresis. The positions of molecular weight (M*r*) standards are indicated on the right, and the *pI* are indicated on the bottom of the gel.

4.2.2.2. Differential protein expression between AAA and non-AAA plasma samples by pooled sample analysis

Of 6 replicates silver-stained 2D-gels of depleted pooled plasma samples, 4 replicates *per* group of gels (AAA and non-AAA) had enough quality to be used for the bioinformatic analysis. Of the 1170 spots detected *per* image, 83 had a p-value lower than 0.05. Of these polypeptide spots, 66

presented a fold-change equal or greater than 1.5. After manual validation, 4 polypeptide spots were selected as differentially expressed between both groups of plasma samples: 1 up-regulated and 3 down-regulated in AAA pooled plasma samples (Table 1).

Table 1. Statistical values of selected spots during the analysis with SameSpots software.

Spot	p (ANOVA)	q Value	Power	Fold Change (AAA *vs.* non-AAA)
18	0.00034	0.26	1	-2.1
44	0.00095	0.26	0.998	1.6
53	0.0021	0.26	0.998	-1.6
66	0.0013	0.26	0.996	-1.5

Fold Change is shown as a mean of normalized volume of the same spot in gels belonging to AAA plasma samples compared to the corresponding values of non-AAA plasma samples.

Preparative 2D-gels were performed under the same conditions than 2D-gel performed for the bioinformatic analysis. However, the 2D-gels obtained had a different proteome profile than the gels used for the differential proteomic analysis (Figure 2).

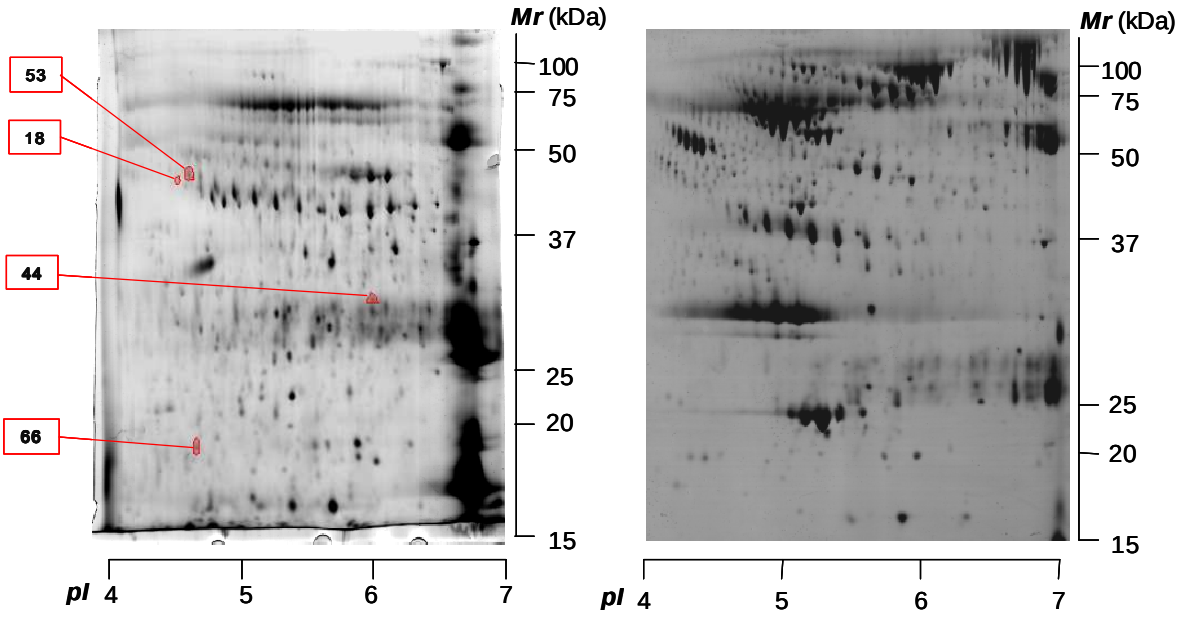


Figure 2. Representative silver-stained 2D-gel of plasma samples. An example of pooled plasma 2D-gel used for the bioinformatic analysis is shown on the left and an example of pooled plasma 2D-gel used for protein identification is presented on the right. A hundred µg of each depleted plasma proteins was used to perform two-dimensional electrophoresis. The positions of molecular weight (M*r*) standards are indicated on the right, and the *pI* are indicated on the bottom of the gel.

Several 2D-gels were performed afterwards but protein profile did not correspond to the protein profile in analytical gels. Thus, proteins corresponding to differentially expressed spots could not be identified by MS analysis.

4.3. nLC-MS/MS analysis of plasma samples

4.3.1. Materials and methods

4.3.1.1. Plasma sample depletion

Plasma samples of 17 AAA patients as well as 17 non-AAA patients, perfectly coupled according to the patients matching, were used for the proteomic analysis. For both groups of patients, a pool was prepared using 50 µL of plasma per patient. Multiple Affinity Removal System Hu-14 spin cartridge (Agilent Technologies, Santa Clara, CA) was used in order to deplete 200 µl of each pool of plasma for the 14 most abundant proteins (albumin, IgG, IgA, IgM, transferrin, fibrinogen, haptoglobin, alpha-1-antitrypsin, apolipoprotein AI, apolipropotein AII, alpha-1-acid glycoprotein, complement C3, transthyretin, alpha-2-macroglobulin) according to the manufacturer's protocol. After depletion, samples were concentrated in 500 µL of 100 mM ammonium bicarbonate using spin concentrators (5k MWCO, catalog no. 5185-5991, Agilent Technologies, Santa Clara, CA). Then, protein concentration was determined by a Bradford assay.

4.3.1.2. Reduction, alkylation, digestion

Urea was added to a volume corresponding to 500 µg of each depleted plasma pool to reach a concentration of 6M. Then, plasma proteins were reduced by incubation with 2.5 µL of 200 mM DTT (dithiothreitol) at 37°C for 1 hr. Next, alkylation was performed by incubation with 11.25 µL of 200 mM IAA (iodoacetamide) at 37°C for 1 hr in the dark. Samples were then diluted three times with 100 mM ammonium bicarbonate before addition of 10 µg of trypsin (enzyme to protein ratio of 1:50). Digestion was carried out overnight at 37ºC.

Peptides of each sample digest were desalted on two C18 macrospin columns (30-300 µg capacity, catalog no. SMM SS18V, The Nest Group, Southborough, MA) according to the manufacturer's instructions, and the two eluates of each sample were pooled. The total volume of each sample, around 300 µL, was splited in four equal volumes and the new tubes were then stored at -80ºC until PAcIFIC mass spectrometry analysis.

The same procedures were performed with 100 µg of each depleted plasma pool for further TMTduplex labeling.

4.3.1.3. TMTduplex labeling

Peptide mixtures corresponding to non-AAA and AAA plasma samples were labeled with TMT^2-126 and TMT^2-127 (ThermoScientific, Rockford, IL), respectively, according to manufacturer's protocol. Briefly, 42 µL of of the TMT label reagent were added to 100 µg of each peptide mixture and incubated for 1 h at room temperature. Then, reaction was stopped by incubation with 8 µL of quenching buffer containing 5% hydroxylamine for 15 min at room temperature. The resulting samples were combined and stored at -80ºC until mass spectrometry analysis.

4.3.1.4. nLC-MS/MS analysis by PAcIFIC

Peptide samples were dried with a vacuum concentrator and resuspended in a buffer containing 5% acetonitril (ACN) and 0.1% formic acid (FA) to reach an estimated peptide concentration of 0.5 µg/µL. HPLC was performed on a Waters NanoAcquity (Manchester, U.K.) system using homemade columns and precolumns and the same chromatographic conditions as previously described [Scherl *et al.* 2008]. An estimated amount of 1.0 µg of plasma peptides was used for each sample injection. An LTQ XL linear ion trap mass spectrometer (ThermoFisher, San Jose, CA) was used to perform PAcIFIC mass spectrometry analysis as previously described [Panchaud *et al.* 2009]. Briefly, in a first injection, PAcIFIC acquisition used 15 consecutive CID MS/MS scans of m/z = 401.5, 403, 404.5, 406, 407.5, 409, 410.5, 412, 413.5, 415, 416.5, 418, 419.5, 421 and 422.5 with an isolation width set to 2.5 and collision energy to 35%. In the consecutive range, m/z values from 422.5 to 445 were analyzed, etc. until m/z = 1412.5 was reached (45 repeated injections in total). Each plasma sample was run in duplicate.

4.3.1.5. PAcIFIC MS for the analysis of TMT-labeled samples

The combined TMTduplex-labeled samples were dried in a vacuum concentrator and dissolved in a buffer containing 5% ACN and 0.1% FA to reach an estimated peptide concentration of 0.5 µg/µL. Mass spectrometer and HPLC conditions were the same described above. PAcIFIC MS analysis was applied as follows: in a first injection, 15 consecutive CID MS/MS scans of m/z = 401.5, 403, 404.5, 406, 407.5, 409, 410.5, 412, 413.5, 415, 416.5, 418, 419.5, 421 and 422.5 with an isolation width set to 2.5 and collision energy to 35%. In between each CID fragmentation scan, a zoomed pulsed-Q dissociation (PQD) scan with collision energy set to 35%, activation Q = 0.55, and activation time = 0.4 was performed from m/z= 121 to 132 in order to measure the TMT reporter ions. In the consecutive range, m/z values from 422.5 to 445 were analyzed by CID and subsequent PQD tandem mass spectrometry, etc. until m/z = 1412.5 was reached (45 repeated injections in total). Each plasma sample was run in duplicate.

4.3.1.6. Data Processing.

For each PAcIFIC replicate, RAW files were converted to mzXML files (45 files in total) and searched with Sequest against the IPI human database v3.49 (http://www.ebi.ac.uk/IPI/IPIhuman.html). The following settings were used for the search: precursor tolerance: 3.75 Da; fixed modification: alkylated cysteines; and variable modification: oxidized methionine. Sequest results were converted to pepXML files and probability assessments of identified peptides were computed with PeptideProphet (ISB, Seattle, WA). For all individual searches (45 pepXML files or 1 replicate), peptides with probability scores equal or higher than 0.9 were used (estimated false discovery rate < 1%). For searches on TMT-labeled samples, the TMT modification was added as fixed and the intensities of TMT reporter ions (m/z= 126 and m/z= 127) were extracted from raw data files, intensity ratios calculated and normalized to 126-TMT reporter ion using the program Libra (ISB, Seattle, WA). For each protein, the ratio was calculated as a mean value of all intensity ratios of peptides identified for that protein.

4.3.1.7. Spectral counting

Only multiple hit proteins (proteins that have two or more identified peptides) were used in spectral count analysis. Datasets of MS/MS data with significant identification assignments were exported into Excel. Number of spectra *per* protein was calculated. Average spectral counts of duplicates were calculated and normalized to the sum of all spectra from the sample with highest number of spectra, i.e. AAA plasma sample. When a protein was identified only in one sample, an arbitrary value of 0.1 was assigned to the number of spectral counts for the other sample. Then, G-test was applied to spectral counts of each protein in every sample in order to evaluate differences of protein expression. P-values lower than 0.5 were considered as significant.

4.3.1.8. Quantitative analysis by TMT isotopic labeling

Only proteins with two or more labeled peptides were used for the quantitative analysis. Datasets of MS/MS data containing mean values of intensity ratios of TMT reporter ions were exported into Excel. Then, t-test was applied to duplicated values of intensity ratios for each protein in order to evaluate differences of protein expression. P-values lower than 0.5 were considered significant.

4.3.1.9. Western blot analysis

Western blot (WB) analysis was performed on the same 17 AAA and 17 non-AAA plasma samples that were used for the proteomic analysis. A plasma sample of a non-AAA patient, that was not included for the proteomic analysis, was loaded in all blots for normalization of results. This

sample is referred as standard sample along the text. Two µL of each plasma sample were diluted with 38 µL of RIPA buffer (10 mM Tris HCl, 150 M NaCl, 10% v/v IGEPAL CA-630 (Sigma Aldrich), 0.5% w/v sodium deoxycholate, 10% w/v SDS, and 10% w/v sodium orthovanadate) and separated by SDS-PAGE (12% acrylamide gels for WB of all proteins except adiponectin for which a 15% acrylamide gel was used). Then, proteins were transferred onto a 0.45 µm Hybond nitrocellulose membrane (GE Healthcare), except for WB of corticosteroid-binging globulin, for which protein plasma samples were transferred onto a polyvinylidine fluoride (PVDF) membrane (Ref. No. 10413096, Sigma Aldrich). Visual verification of total protein loads was performed by Ponceau staining of the membranes. For PVDF membranes, this step was performed at the end of the protocol. Ponceau stained membranes were scanned and washed with TBS-Tween for 5 min twice at room temperature, then saturated with 5% w/v non fat dry milk TBS-Tween for 45 min at room temperature, and incubated with blocking buffer containing primary antibodies against specific proteins at 4ºC, overnight. Eight primary antibodies were used: polyclonal goat anti human coagulation factor XII (1:1000 v/v dilution, Ref. No.: LS-C23414-100, Life span biosciences); polyclonal rabbit anti human adiponectin (1:1000 v/v dilution, Ref. No.: ab13881, Abcam); monoclonal mouse anti human gelsolin (1:5000 v/v dilution, Ref. No.: G4896, Sigma Aldrich); polyclonal rabbit anti human corticosteroid-binding globulin (1:500 v/v dilution, Ref. No.: 12010-1-AP, Proteintech group inc); polyclonal rabbit anti human extracellular superoxide dismutase [Cu-Zn] (1:100 v/v dilution, Ref. No.: R-170-100, NovusBiological); polyclonal goat anti human kallistatin (1:2000 v/v dilution, Ref. No.: AF1669, R&D systems); monoclonal mouse anti human carboxypeptidase B2 (1:1000 v/v dilution, Ref.No.: ab14757, Abcam); and polyclonal rabbit anti human alpha-1-antitrypsin (1:10000 v/v dilution, Ref. No.: A001202, Dako). After incubation with primary antibodies, membranes were washed in TBS-Tween for 10 min, 3 times, and incubated with blocking solution containing 1:5000 v/v secondary antibodies: donkey anti goat (Ref. No.: SC-2020, Abcam), ECL mouse IgG-HRP (Ref. No.: NA931, GE Healthcare), and ECL rabbit IgG-HRP (Ref. No.: NA934, GE Healthcare) for 1.5 h. Finally, membranes were washed with TBS-Tween for 10 min, 3 times, and then incubated with ECL Plus western blotting detection reagent (Ref. No.: RPN2132, GE Healthcare) for 1 min. Blot detection was performed by membrane scanning with an Ettan DIGE Imager scanner (GE Healthcare) at excitation/emission wavelengths of 480 nm/530 nm to yield images with a pixel size of 100 µm.

Quantity One software (Biorad) was used for the acquisition of intensity values of detected proteins from blot images. For the quantification of each protein, images of Ponceau-stained membranes as well as fluorescent images were used. For each protein, a rectangle was defined and used to obtain intensity values from detected bands in *n* plasma samples (WB_n) and in the standard sample (StWB) of every membrane. The corresponding background intensity (B_n) was also measured

for each band. An arbitrary band was selected in Ponceau images and intensity values were measured in plasma samples (RP_n) and in the standard sample (StRP). For each protein, intensity values were exported into Excel and quantification was performed following several calculation steps: 1) for each membrane, Ponceau values of each plasma sample were normalized to the standard sample (RP_n/StRP=NRP_n), 2) background intensity was subtracted from intensity values corresponding to the protein band that was analyzed (WB_n-B_n=BWB_n), 3) background subtracted intensity values were normalized to the corresponding Ponceau image (BWB_n/NRP_n=NWB_n), and 4) then to the standard sample (NWB_n/StWB= Q_n) to finally obtain a normalized (inter and intra membrane) intensity value for the protein to be quantified in the *n* plasma samples (Q_n). Permutation test for non parametric inference was used to evaluate differences between protein expression levels in AAA samples compared to non-AAA samples.

4.3.2. nLC MS/MS analysis of plasma samples

4.3.2.1. Protein analysis by PAcIFIC MS analysis

Thirty four plasma samples of patients included in CORONA clinical protocol (17 AAA patients and 17 non-AAA patients) were analyzed by a recent powerful MS method termed PAcIFIC for Precursor Acquisition Independent From Ion Count. Before PAcIFIC MS analysis, samples were pooled in two groups (AAA and non-AAA) and depleted for the 14 most-abundant proteins (Figure 1).

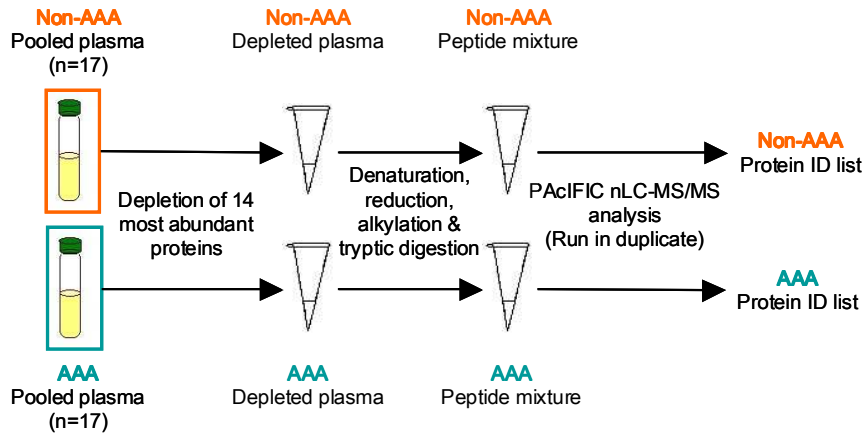


Figure 1. Workflow for the proteomic analysis of protein plasma samples. For each group of patients, 17 plasma samples were pooled and depleted for the 14 most-abundant proteins. Then, after denaturation, reduction, and alkylation, proteins were digested with trypsin and peptide mixtures were analyzed in duplicate by PAcIFIC MS method.

Considering only peptide identifications with probability scores equal or higher than 0.9 (false discovery rate (FDR) < 1%), PAcIFIC MS analysis allowed identification of around one thousand

proteins *per* sample. In non-AAA pooled plasma sample, an average of 328 proteins were identified with at least 2 peptides (multiple hit identification), whereas 667 proteins were identified with only one peptide (single hit identification). In AAA pooled plasma sample, an average of 311 proteins were identified as multiple hits while 687 proteins were identified as single hits. From multiple hit identified proteins, known plasma protein concentration values, described by the HUPO Plasma Proteome Project [Haab *et al.* 2005], were used to calculate dynamic range. PAcIFIC MS analysis provided a dynamic range of 4.5E+06 for protein identification in plasma samples that were depleted for the 14 most abundant proteins (only proteins identified as multiple hits, with false discovery rate lower than 1%, were considered). Table 1 presents a list of representative proteins which concentration in plasma has been described.

Table 1. List of representative identified proteins with known concentration in plasma

Accession number (UniProtKB)	Protein name	Concentration in plasma [pg/mL]
P08253	72 kDa type IV collagenase	8.8E+03
Q58P21	Metalloproteinase inhibitor 1	1.4E+04
P18065	Insulin-like growth factor-binding protein 2	1.5E+04
Q13740	Isoform 1 of CD166 antigen	1.6E+04
Q8WW79	L-selectin	1.7E+04
P33151	Cadherin-5	3.0E+04
P05362	Intercellular adhesion molecule 1	4.3E+04
P17936	Insulin-like growth factor-binding protein 3	5.9E+04
A8K6R7	Isoform 1 of Vascular cell adhesion protein 1	9.4E+04
P11226	Mannose-binding protein C	9.7E+04
P61769	Beta-2-microglobulin	1.1E+06
P04275	von Willebrand factor	1.3E+06
P02649	Apolipoprotein E	3.4E+07
P02751	Isoform 1 of Fibronectin	1.1E+08
P00747	Plasminogen	1.4E+08
P0C0L4	Complement C4-A	1.7E+08
P00450	Ceruloplasmin	2.1E+08
P02766	Transthyretin	2.6E+08
P02652	Apolipoprotein A-II	3.0E+08
Q7KZ97	Antithrombin III variant	3.2E+08
P02763	Alpha-1-acid glycoprotein 1	6.1E+08
P19652	Alpha-1-acid glycoprotein 2	6.1E+08
P02671	Isoform 1 of Fibrinogen alpha chain	6.7E+08

P02675	Fibrinogen beta chain	6.7E+08
P04114	Apolipoprotein B-100	7.2E+08
P02790	Hemopexin	7.5E+08
A7E236	Complement C3 (Fragment)	9.5E+08
P01009	Isoform 1 of Alpha-1-antitrypsin	1.1E+09
P01023	Alpha-2-macroglobulin	1.4E+09
P02647	Apolipoprotein A-I	1.4E+09
P02787	Serotransferrin	2.3E+09
A6NBZ8	Albumin	4.0E+10
	Dynamic range:	4.5E+06

Dynamic range of protein identification provided by PAcIFIC was calculated according to the concentration values of proteins in plasma described by the HUPO Plasma Proteome Project [Haab *et al.* 2005]. This methodology provided a dynamic range of 4.5E+06 when only proteins identified as multiple hits, with false discovery rate lower than 1%, were considered.

4.3.2.2. Protein quantification by spectral counting

Proteins identified as multiple hits (at least two unique peptides *per* protein identified) were quantified by spectral counting. Significant differences were evaluated by the application of G-test. Of 360 proteins quantified in total, 52 proteins appeared to be significantly differentially expressed between non-AAA and AAA pooled plasma samples (Table 2). Fifteen of them (adenylyl cyclase-associated protein 1; pleckstrin; talin-1; profilin-1; alpha-actinin-1; filamin-A; actin, aortic smooth muscle; vinculin; alpha-2-antitrypsin; carboxypeptidase N catalytic chain; gelsolin; heparin cofactor 2; AMBP protein; alpha-1-antitrypsin; and extracellular superoxide dismutase [Cu-Zn]) were selected for further validation by Western blot analysis. These proteins were selected according to their implication in aneurismal formation and evolution. Alpha-1-antitrypsin belonged to the 14 most abundant proteins that were depleted in the first step of the proteomic analysis. Despite this fact, it was also selected for further validation because of its importance on platelet activation and intraluminal thrombus formation mechanisms.

Table 2. Spectral count quantitative analysis between plasma proteins from aneurismal patients and non-aneurismal patients.

IPI	Accession number	Protein name	p (G-test)	Ratio AAA *vs.* Non-AAA
IPI00384952	Q7Z379*	Putative Uncharacterized protein DKFZp686K04218 (Fragment)	0.027	0.02
IPI00061977	-	IGHA1 protein	8.0E-06	0.1

IPI00215983	P00915	Carbonic anhydrase 1	4.6E-04	0.1
IPI00008274	**Q01518**	**Adenylyl cyclase-associated protein 1**	**0.002**	**0.1**
IPI00306311	**P08567**	**Pleckstrin**	**0.007**	**0.1**
IPI00163446	P01880	Isoform 2 of Ig delta chain C region	0.031	0.1
IPI00298994	**Q9Y490**	**Talin-1**	**3.7E-07**	**0.2**
IPI00019359	P35527	Keratin, type I cytoskeletal 9	2.8E-04	0.2
IPI00216691	**P07737**	**Profilin-1**	**0.029**	**0.2**
IPI00220327	P04264	Keratin, type II cytoskeletal 1	5.3E-05	0.3
IPI00029168	P08519	Apolipoprotein(a)	3.0E-04	0.3
IPI00009865	P13645	Keratin, type I cytoskeletal 10	0.014	0.3
IPI00180240	A8MW06	Thymosin beta-4-like protein 3	0.037	0.3
IPI00013508	**P12814**	**Alpha-actinin-1**	**0.046**	**0.3**
IPI00021439	P60709	Actin, cytoplasmic 1	1.8E-07	0.4
IPI00302592	**P21333-2**	**Isoform 2 of Filamin-A**	**5.0E-04**	**0.4**
IPI00008603	**P62736**	**Actin, aortic smooth muscle**	**0.004**	**0.4**
IPI00291175	**P18206**	**Isoform 1 of Vinculin**	**0.036**	**0.5**
IPI00154742	Q8N355*	IGL@ protein	0.025	0.6
IPI00029863	**P08697**	**Alpha-2-antitrypsin**	**9.6E-07**	**0.7**
IPI00010295	**P15169**	**Carboxypeptidase N catalytic chain**	**0.026**	**0.7**
IPI00025204	O43866	CD5 antigen-like	0.049	0.7
IPI00032328	P01042	Isoform HMW of Kininogen-1	7.9E-05	0.8
IPI00026314	**P06396**	**Isoform 1 of Gelsolin**	**0.001**	**0.8**
IPI00292950	**P05546**	**Heparin cofactor 2**	**0.003**	**0.8**
IPI00294395	P07358	Complement component C8 beta chain	0.004	0.8
IPI00021854	P02652	Apolipoprotein A-II	0.011	0.8
IPI00292530	P19827	Inter-alpha-trypsin inhibitor heavy chain H1	6.6E-05	0.9
IPI00550991	P01011	SERPINA3	1.2E-04	0.9
IPI00029739	P08603	Isoform 1 of Complement factor H	0.001	0.9
IPI00022431	P02765	Alpha-2-HS-glycoprotein	0.002	0.9
IPI00305461	P19823	Inter-alpha-trypsin inhibitor heavy chain H2	0.007	0.9
IPI00022418	P02751	Isoform 1 of Fibronectin	0.008	0.9
IPI00017696	P09871	Complement C1s subcomponent	0.033	0.9
IPI00017601	P00450	Ceruloplasmin	7.5E-06	1.1
IPI00022229	P04114	Apolipoprotein B-100	4.1E-04	1.1
IPI00218192	Q14624	Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H4	0.001	1.1
IPI00032258	P0C0L4	Complement C4-A	4.3E-13	1.2
IPI00022895	P04217	Alpha-1B-glycoprotein	4.1E-05	1.2

IPI00478003	P01023	Alpha-2-macroglobulin	0.006	1.2
IPI00022426	**P02760**	**AMBP protein**	**0.009**	**1.2**
IPI00879709	-	Complement component 6 precursor	0.034	1.2
IPI00783987	P01024	Complement C3 (Fragment)	0.011	1.3
IPI00025426	P20742	Isoform 1 of Pregnancy zone protein	0.035	1.3
IPI00418163	Q6P4R1*	Complement component 4B preproprotein	3.3E-05	1.4
IPI00028413	Q06033	Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H3	8.7E-05	1.4
IPI00021856	P02655	Apolipoprotein C-II	0.015	1.4
IPI00553177	**P01009**	**Isoform 1 of Alpha-1-antitrypsin**	**0.020**	**1.4**
IPI00022417	P02750	Leucine-rich alpha-2-glycoprotein	0.028	1.4
IPI00893864	B0UXW4*	Complement factor B	0.026	2.5
IPI00027827	**P08294**	**Extracellular superoxide dismutase [Cu-Zn]**	**0.041**	**4.7**
IPI00011302	P13987	CD59 glycoprotein	0.005	6.9

Proteins that were found to be differentially expressed by spectral count quantitative analysis. G-test was applied to analyze differential expression of plasma proteins. Proteins with $p < 0.05$ were selected as significantly differentially expressed. Assignment of UniProtKB accession numbers were made according to UniProt Knowledgebase Release 15.8 of Sep 22, 2009. * accession number refers to UniProtKB/TrEMBL database instead of UniProtKB/Swiss-Prot. In bold highlighted: proteins selected for further validation by Western blot.

4.3.2.3. Complementary quantification analysis by TMT isobaric labeling

Pooled plasma samples of AAA and non-AAA patients were also quantified in duplicate by an isobaric labeling strategy showed in Figure 2.

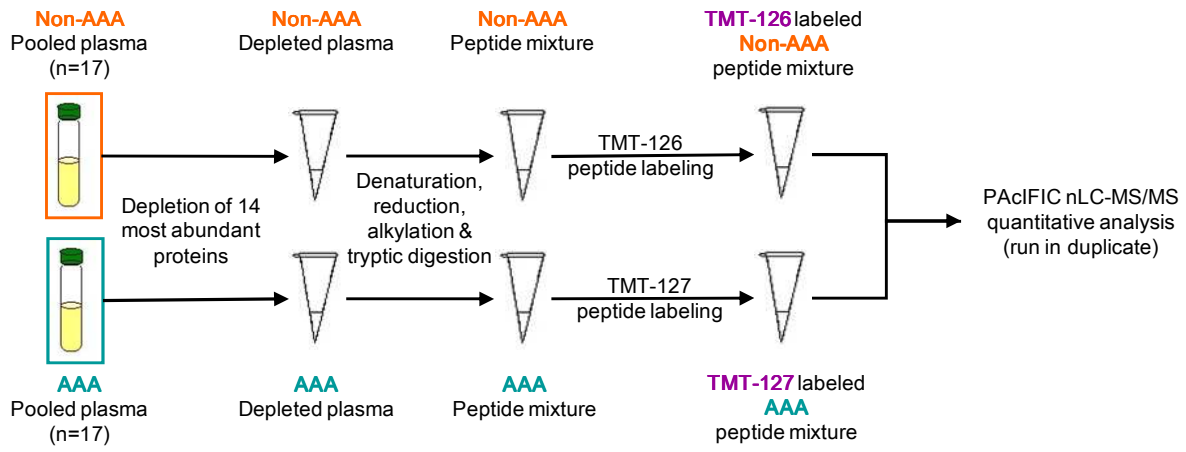


Figure 2. Workflow for TMT quantitative analysis of protein plasma samples. For each group of patients, 17 plasma samples were pooled and depleted for the 14 most-abundant proteins. Then, after denaturation, reduction, and alkylation, proteins were digested with trypsin and isotopic labeling with TMT-126 and TMT-127

tags was performed. Labeled peptides were mixed and analyzed in duplicate by PAcIFIC MS method and intensity ratios of reporter ions were used to perform relative quantification of the proteins.

Of an average number equal to 788 proteins that were identified, 147 proteins had, at least, two peptides for which both 126-TMT and 127-TMT reporter ions could be detected and intensity values were used to calculate the AAA to non-AAA protein ratios. T-test was applied to duplicated values of intensity ratios and, with a p-value < 0.05, 65 proteins appeared to be differentially expressed between AAA and non-AAA pooled plasma samples (Table 3). Only 15 of the differentially expressed proteins were also found to be differentially expressed by spectral count analysis. Corresponding ratios to each quantitative method are shown in Table 3. Six proteins (alpha-2-antitrypsin, inter-alpha-trypsin inhibitor heavy chain H1, highly similar to alpha-1-antichymotrypsin, isoform 1 of complement factor H, alpha-2-HS-glycoprotein, and complement C1s subcomponent) showed up-regulation in AAA pooled plasma samples when quantification was performed by TMT labeling while they showed under-regulation in AAA pooled samples when quantification was performed by spectral count. For the other nine proteins, the sense of the regulation in both quantitative analyses appeared to be the same.

From this analysis, 7 additional proteins that could be involved in aneurismal pathology (platelet basic protein, corticosteroid binding globulin, kallistatin, coagulation factor XII, carboxypeptidase B2, lysozyme C, and adiponectin) were selected for further validation with western blot analysis.

Table 3. TMT quantification of plasma proteins between aneurismal patients and non-aneurismal patients.

IPI	Accession number	Protein name	TMT-labeling ratio	Spectral count ratio
			AAA *vs.* Non-AAA	**AAA *vs.* Non-AAA**
IPI00027843	P22891	Isoform 1 of Vitamin K-dependent protein Z	0.5	-
IPI00022445	**P02775**	**Platelet basic protein**	**0.7**	**-**
IPI00654755	P68871	Hemoglobin subunit beta	0.8	-
IPI00909594	B4E3S6*	Highly similar to Complement component C7	0.8	-
IPI00019399	P35542	Serum amyloid A-4 protein	0.8	-
IPI00027482	**P08185**	**Corticosteroid-binding globulin**	**0.8**	**-**
IPI00001611	P01344	Isoform 1 of Insulin-like growth factor II	0.8	-
IPI00021854	P02652	Apolipoprotein A-II	0.8	0.8
IPI00328609	**P29622**	**Kallistatin**	**0.9**	**-**

IPI00294004	P07225	Vitamin K-dependent protein S	0.9	-
IPI00022731	P55056	Apolipoprotein C-IV	0.9	-
IPI00029061	P49908	Selenoprotein P	0.9	-
IPI00020996	P35858	Highly similar to Insulin-like growth factor-binding proteincomplex acid labile chain	0.9	-
IPI00641737	P00738	Haptoglobin	0.9	-
IPI00292950	P05546	Heparin cofactor 2	0.9	0.8
IPI00021817	P04070	Vitamin K-dependent protein C	1.0	-
IPI00019581	**P00748**	**Coagulation factor XII**	**1.1**	**-**
IPI00022391	P02743	Serum amyloid P-component	1.1	-
IPI00414283	P02751	Fibronectin 1 isoform 4 preproprotein	1.1	-
IPI00019943	P43652	Afamin	1.1	-
IPI00218732	P27169	Serum paraoxonase/arylesterase 1	1.1	-
IPI00296165	B4DPQ0*	Highly similar to Complement C1r subcomponent	1.1	-
IPI00032291	P01031	Complement C5	1.1	-
IPI00022463	P02787	Serotransferrin	1.1	-
IPI00296608	P10643	Complement component C7	1.1	-
IPI00032179	P01008	Antithrombin III variant	1.1	-
IPI00022420	P02753	Retinol-binding protein 4	1.1	-
IPI00029863	P08697	Alpha-2-antiplasmin	1.1	0.7
IPI00292530	P19827	Inter-alpha-trypsin inhibitor heavy chain H1	1.1	0.9
IPI00550991	P01011	Highly similar to Alpha-1-antichymotrypsin	1.1	0.9
IPI00029739	P08603	Isoform 1 of Complement factor H	1.1	0.9
IPI00022431	P02765	Alpha-2-HS-glycoprotein	1.1	0.9
IPI00017696	P09871	Complement C1s subcomponent	1.1	0.9
IPI00022895	P04217	Alpha-1B-glycoprotein	1.1	1.2
IPI00879709	P13671	Complement component 6	1.1	1.2
IPI00022395	P02748	Complement component C9	1.2	-
IPI00022488	P02790	Hemopexin	1.2	-
IPI00298971	P04004	Vitronectin	1.2	-
IPI00029658	Q12805	Isoform 1 of EGF-containing fibulin-like extracellular matrix protein 1	1.2	-
IPI00006114	P36955	Pigment epithelium-derived factor	1.2	-
IPI00021855	P02654	Apolipoprotein C-I	1.2	-
IPI00896419	Q14624	Inter-alpha (globulin) inhibitor H4	1.2	-
IPI00029260	P08571	Monocyte differentiation antigen	1.2	-

		CD14		
IPI00022392	P02745	Complement C1q subcomponent subunit A	1.2	-
IPI00021727	P04003	C4b-binding protein alpha chain	1.2	-
IPI00019591	P00751	Highly similar to Complement factor B	1.2	-
IPI00011264	Q03591	Complement factor H-related protein 1	1.2	-
IPI00292946	P05543	Thyroxine-binding globulin	1.2	-
IPI00017601	P00450	Ceruloplasmin	1.2	1.1
IPI00022229	P04114	Apolipoprotein B-100	1.2	1.1
IPI00478003	P01023	Alpha-2-macroglobulin	1.2	1.2
IPI00303963	P06681	Complement C2	1.3	-
IPI00006543	Q9BXR6	Complement factor H-related 5	1.3	-
IPI00021857	P02656	Apolipoprotein C-III	1.3	-
IPI00299503	P80108	Isoform 1 of Phosphatidylinositol-glycan-specific phospholipase D	1.3	-
IPI00329775	**Q96IY4**	**Isoform 1 of Carboxypeptidase B2**	**1.3**	**-**
IPI00028413	Q06033	Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H3	1.3	1.4
IPI00553177	P01009	Isoform 1 of Alpha-1-antitrypsin	1.3	1.4
IPI00022394	P02747	Complement C1q subcomponent subunit C	1.4	-
IPI00021885	P02671	Isoform 1 of Fibrinogen alpha chain	1.4	-
IPI00021891	P02679	Isoform Gamma-B of Fibrinogen gamma chain	1.4	-
IPI00019038	**P61626**	**Lysozyme C**	**1.4**	**-**
IPI00167093	Q03591	Complement factor H-related protein 1	1.5	-
IPI00165972	P00746	Complement factor D preproprotein	1.7	-
IPI00020019	**Q15848**	**Adiponectin**	**1.8**	**-**

Proteins that were found to be differentially expressed specifically by TMT-labeling approach. T-test was applied to analyze differential expression of plasma proteins. Proteins with $p < 0.05$ were selected as significantly differentially expressed. Assignment of UniProt accession numbers were made according to UniProt Knowledgebase Release 15.8 of Sep 22, 2009. * accession number refers to UniProt/TrEMBL database instead of UniProt/Swiss-Prot. In bold highlighted: proteins selected for further validation by Western blot.

4.3.2.4. Western blot validation of differentially regulated proteins

In all, 22 proteins were selected from quantitative proteomic analyses to be validated by Western blot analysis. These proteins could be classified in three categories depending on their relation with AAA. First, proteins involved in actin reorganization: adenylyl cyclase-associated protein

1; talin-1; profilin-1; alpha-actinin-1; isoform 2 of filamin-A; actin, aortic smooth muscle; vinculin; and gelsolin. These proteins may be implicated in vascular remodeling. Second, proteins involved in platelet activation and intraluminal thrombus formation: alpha-2-antitrypsin; pleckstrin; carboxypeptidase N catalytic chain; heparin cofactor 2; AMBP protein; isoform 1 of alpha-1-antitrypsin; extracellular superoxide dismutase, platelet basic protein, corticosteroid binding globulin, kallistatin, coagulation factor XII, and carboxypeptidase B2. Finally, lysozyme C, and adiponectin, are involved in several biological processes including inflammatory response.

Table 4. Comparison between protein ratios obtained by all three quantitative analyses.

Protein	SC ratio AAA *vs.* Non-AAA	TMT ratio AAA *vs.* Non-AAA	Quantified band	WB ratio AAA *vs.* Non-AAA	p value (WB)
Gelsolin	0.8	-	-	1.2	0.67
Adiponectin	-	1.8	both bands	1.7	**0.0092**
			upper band	1.6	**0.033**
			lower band	1.7	**0.036**
Alpha-1-antitrypsin	1.4	1.3	-	1.1	0.069
Extracellular superoxide dismutase	4.7	-	-	1.6	**0.007**
Corticosteroid-binding globulin	-	0.8	both bands	0.8	0.25
			upper band	0.8	0.23
			lower band	1	0.73
Kallistatin	-	0.9	both bands	1.3	0.11
			upper band	1.1	0.47
			lower band	1.3	**0.046**
Coagulation factor XII	-	1.1	-	1	0.96
Carboxypeptidase B2	-	1.3	-	1.6	**0.0061**

SC: spectral count analysis. TMT: isobaric tagging approach. WB: western blot analysis. When several bans were detected on a western blot, quantification was performed taking account of all bands and for each separated band, protein ratios found in all cases are shown. In bold highlighted: significant p values for permutation test.

Western blot analysis for all proteins was performed on diluted plasma samples, but only 8 proteins (gelsolin, adiponectin, alpha-1-antitrypsin, extracellular superoxide dismutase, corticosteroid-binding globulin, kallistatin, coagulation factor XII, and carboxypeptidase B2) could be detected and quantified (Figure 3). When protein was detected in more than one band, quantification was performed for each separated band and for both together. Four of these proteins presented different expression between AAA and non-AAA plasma samples: adiponectin, carboxypeptidase B2, kallistatin, and extracellular superoxide dismutase. AAA to non-AAA ratios of both proteomic and western blot quantification are presented in Table 4.

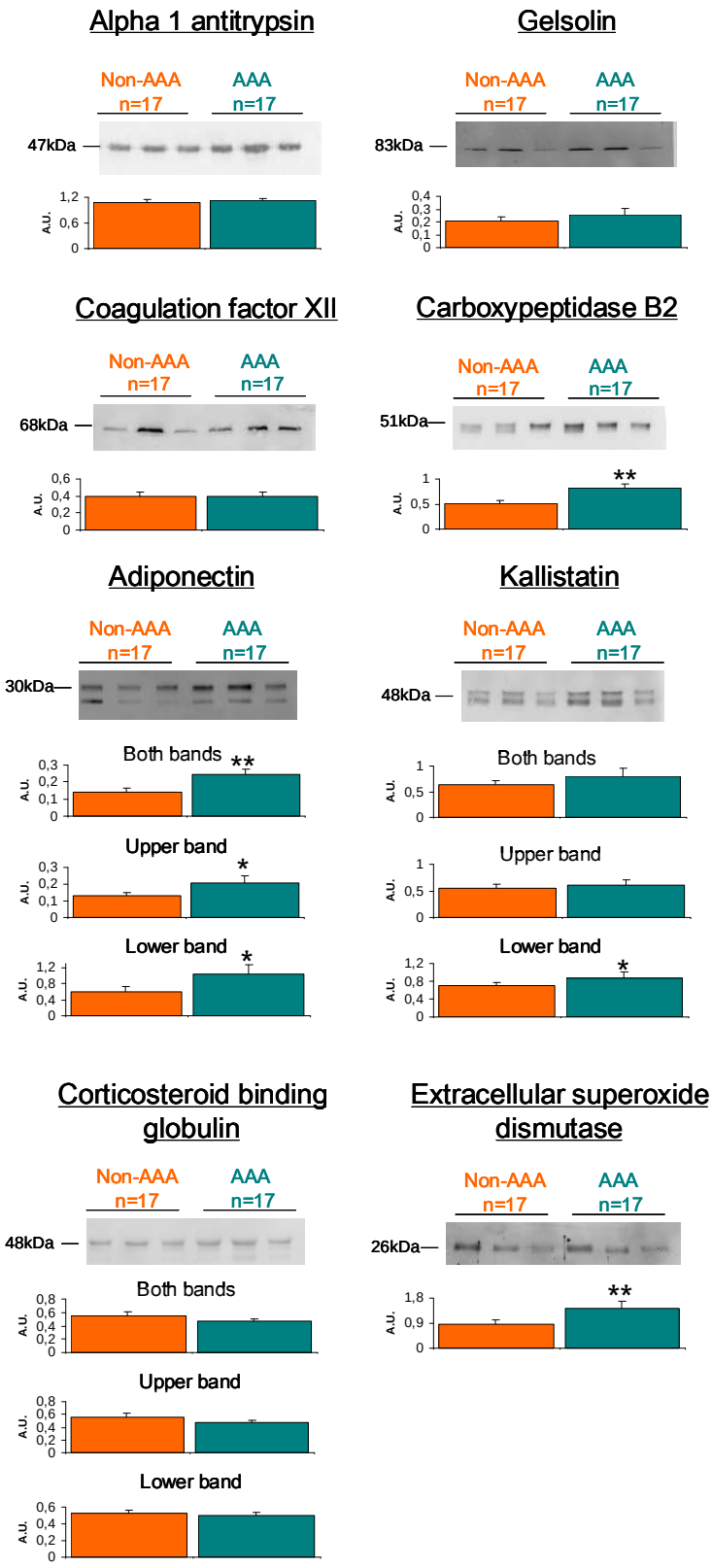


Figure 3. Western blot analysis of selected proteins for validation of MS/MS quantitative results between plasma samples of aneurismal and non-aneurismal patients. M*r* values for each protein are indicated on the left. Data was expressed as mean of the arbitrary units (A.U.) ± SEM. Permutation test for non-parametric inference was applied. * $p<0.05$; ** $p<0.01$

4.3.3. Discussion on nLC MS/MS analysis of plasma samples

4.3.3.1. Comparison between both quantitative MS approaches

The aim of this part of the study, which was performed in collaboration with the laboratory of Pr. Goodlett, in Seattle (USA), was to use differential proteomic analysis to analyze plasma samples of 17 patients having AAA and compare them to 17 plasma samples of non-AAA patients, in order to find differentially expressed proteins that may be potential biological markers for AAA diagnosis. Plasma samples were pooled and depleted for the 14 most abundant proteins. Then, two different mass spectrometry based approaches were applied on depleted samples.

The first approach combined the use of PAcIFIC MS analysis [Panchaud *et al.* 2009] with spectral count [Liu *et al.* 2004]. PAcIFIC is a recent data independent method that has been developed in the laboratory of Pr. Goodlett, in which no precursor ions are analyzed before MS/MS fragmentation. Since there is no selection of precursor ion, tandem mass analysis is performed sequentially in a way that allows coverage of a m/z range from 400 to 1400. Spectral count was applied on data independent MS data in order to assess differential protein expression between both groups of plasma samples (AAA *vs.* non-AAA). Three hundred and sixty proteins that covered dynamic range of 4.5E+6 were evaluated. For all these proteins, two or more peptides were identified. Among 52 proteins that appeared to be differentially expressed between both groups of plasma samples, 15 (adenylyl cyclase-associated protein 1; pleckstrin; talin-1; profilin-1; alpha-actinin-1; filamin-A; actin, aortic smooth muscle; vinculin; alpha-2-antitrypsin; carboxypeptidase N catalytic chain; gelsolin; heparin cofactor 2; AMBP protein; alpha-1-antitrypsin; and extracellular superoxide dismutase [Cu-Zn]) were selected for further validation by Western blot according to their possible relationship with aneurismal development, i.e. proteins involved in actin reorganization that could give rise to vascular remodeling, and proteins involved in intraluminal thrombus formation since its volume is correlated with the severity of aortic dilation [Golledge *et al.* 2008c].

The second approach combined the use of data independent MS analysis with TMT isobaric labeling. At the time of its application, this method was still under optimization. The TMT duplex label reagent set is composed of two isobaric tags that generate reporter ions at different m/z values, i.e. 126 and 127. Thus, non-AAA plasma samples were labeled with 126-TMT reagent and AAA plasma samples were labeled with 127-TMT reagent. This time, 147 proteins were evaluated for differential expression levels between AAA and non-AAA plasma samples. These proteins contained, at least, two peptides for which both TMT reporter ions were detected and protein ratios could be calculated. Of 65 proteins that appeared to be differentially expressed between both groups of

plasma samples, 7 proteins (platelet basic protein, corticosteroid binding globulin, kallistatin, coagulation factor XII, carboxypeptidase B2, lysozyme C, and adiponectin) were additionally selected, following the same criteria than in spectral count analysis, to be validated by Western blot analysis.

Regarding both MS quantitative approaches, only 15 significantly differentially expressed proteins were common to both methodologies. That represented for each one, less than 30% of differentially expressed proteins. These results suggest that label-free and label-based approaches may be considered as orthogonal, since the information obtained is quite different. Indeed, sample processing for the isobaric tagging approach took more steps, possibly involving loose of protein. Moreover, chemical derivatization of peptides implies modification of their physico-chemical properties, and subsequent modification of peptide behavior during ionization and fragmentation in MS analysis. Interestingly, six of the common proteins (alpha-2-antitrypsin, inter-alpha-trypsin inhibitor heavy chain H1, highly similar to alpha-1-antichymotrypsin, isoform 1 of complement factor H, alpha-2-HS-glycoprotein, and complement C1s subcomponent) showed over-regulation in AAA pooled plasma samples when quantification was performed by TMT labeling while they showed under-regulation in AAA pooled samples when quantification was performed by spectral count. Protein ratios went from 0.9 to 1.1 in all of them except alpha-2-antittrypsin for which spectral count ratio was 0.7. Thus, these ratios correspond to values close to 1 and slight differences during sample processing could affect peptide concentration and produce differences on the sense of the regulation. Moreover, due to instrument time consumption, one sample replicate was analyzed in duplicate by both methodologies. An increase on the number of both sample and technical replicates may help to increase the accuracy of protein ratios in both techniques.

4.3.3.2. Validation of differential expression of selected proteins

From both quantitative MS analysis, 22 proteins were selected to be validated by Western blot analysis, which was performed separately on the same individual plasma samples that were pooled for the proteomic analysis. Usually, for detection of biomarkers, plasma or serum samples are directly analyzed by ELISA or other quantitative biochemical assays, so in order to reproduce the clinical situation, depletion of the 14 most abundant plasma proteins was not performed before Western blot analysis. Unfortunately, only 8 proteins (gelsolin, adiponectin, alpha-1-antitrypsin, extracellular superoxide dismutase, corticosteroid-binding globulin, kallistatin, coagulation factor XII, and carboxypeptidase B2) could be detected and quantified, suggesting that protein concentration enrichment by depletion of the most abundant proteins in plasma was a critical step that allowed an increase of sensitivity during MS analysis. Consequently, Western blot analysis on depleted plasma samples would be required in order to validate differential protein expression of the other 14 proteins (adenylyl cyclase-associated protein 1, talin-1, profilin-1, alpha-actinin-1, isoform 2 of

filamin-A, actin aortic smooth muscle, vinculin, alpha-2-antitrypsin, pleckstrin, carboxypeptidase N catalytic chain, heparin cofactor 2, AMBP protein, platelet basic protein, and lysozyme) that appeared to be differentially expressed by MS quantitative analysis.

Adiponectin, extracellular superoxide dismutase, kallistatin, and carboxypeptidase B2 were significantly differentially expressed by Western blot analysis. AAA to non-AAA ratio for adiponectin was 1.8 when quantitative analysis was performed by TMT-labeling approach. By Western blot analysis 2 bands very close by their molecular weight (≈ 30 kDa) were detected. This could be explained by the presence of a signal peptide at the N-terminal side of the protein which molecular mass corresponds to 1886 Da, which could be removed in the corresponding lighter band. AAA to non-AAA ratios for both heavy and light adiponenctin bands were respectively 1.6 and 1.7, values that are in accordance to MS quantification. Thus, adiponectin appeared to be over expressed in plasma samples from AAA patients compared to non-AAA patients recruited in CORONA clinical protocol. Adiponectin is a cytokine released by adipose tissue. This protein has been described to have anti-diabetic, anti-atherogenic, and anti-inflammatory activities [Ouchi *et al.* 2003]. Indeed, it has been described that diabetic patients have lower levels of plasma adiponectin than non-diabetic patients [Karakitsos *et al.* 2006]. It is also well known that diabetes is negatively associated with abdominal aortic aneurysm [Lederle *et al.* 1997a; Wanhainen *et al.* 2005; Golledge *et al.* 2006]. Moreover, a recent study by Cai *et al.* [Cai *et al.* 2008] led to the hypothesis that adiponectin may be released by adipose tissue around adventitia, and reduce oxidative/nitrative stress to finally mediate anti-atherosclerosis through the stabilization of the plaque. Furthermore, adiponectin strongly suppresses aortic SMC proliferation and migration [Arita *et al.* 2002], one of the key factors in atherosclerosis development. However, AAA is characterized by an increased SMC depletion from the media layer of the aortic wall [Thompson *et al.* 1997; Henderson *et al.* 1999]. Furthermore, adiponectin has been described to selectively increase TIMP-1 expression in human monocyte-derived macrophages [Kumada *et al.* 2004], and TIMP-1 appeared to be correlated with MMP-2 and MMP-9 expression in human AAA biopsies [Nishimura *et al.* 2003], suggesting that it is importantly involved in the developmental process of AAA. Taken all together, it seems that over expression of adiponectin in AAA may help to explain the negative association between diabetes and AAA. Also, adiponectin seems to play an important role in SMC depletion as well as in matrix degradation through the increase expression of TIMP-1. Increased level of serum adiponectin in AAA patients was already described by Golledge *et al.* [Golledge *et al.* 2007a]. In this study, the association between anthropometric measurements of obesity, serum adipokines and AAA was assessed. Interestingly, serum adiponectin was significantly associated with AAA ≥30 mm but not with AAA ≥40 mm. The

results of that study may be interpreted like adiponectin could be useful to discriminate AAA at the first stage of development but not to follow AAA progression.

AAA to non-AAA ratio for extracellular superoxide dismutase (EC-SOD) was 4.7 when quantitative analysis was performed by spectral count analysis and 1.6 when quantitative analysis was performed by Western blot. Thus, over expression of EC-SOD in plasma samples from AAA patients compared to non-AAA patients was validated in patients recruited in CORONA clinical protocol. EC-SOD is a metalloenzyme that requires the presence of Cu and Zn to catalyze the conversion of peroxide radicals into hydrogen peroxide and oxygen to protect the extracellular space from ROS, so it can be classified as an antioxidant enzyme. Interestingly, studies on an elastase induced rat model of AAA, in which quantitative real-time PCR was used to quantify EC-SOD expression, showed that EC-SOD expression did not vary between aortic tissue of sham- and AAA-rats [Sinha *et al.* 2007]. Furthermore, a transcriptomic analysis, also on this elastase induced rat model of AAA, revealed down-regulation of antioxidant genes such as EC-SOD (fold change: –21 at day 10 after elastase perfusion) during AAA formation [Yajima *et al.* 2002]. These results were previously described in human studies in which, despite the fact that Cu and Zn levels in human AAA and athero occlusive diseases (AOD) tissues were similar to the level in control tissues, the activity of EC-SOD in human AAA and AOD aortic tissues was significantly lower than in control aortic tissues [Dubick *et al.* 1987; Dubick *et al.* 1999]. Thus, EC-SOD expression in AAA tissue is lower than in control tissue whereas EC-SOD expression in plasma samples from AAA patients is higher than in plasma samples from non-AAA patients. These results suggest a possible equilibrium between the level of EC-SOD in aortic tissue and the level of EC-SOD secreted into the blood stream. There are evidences for increased levels of ROS and, consequently, an increased level of oxidative stress in human AAA [Miller, Jr. *et al.* 2002]. A decreased amount of antioxidant proteins within aneurismal tissue may be behind this observation and may be reflected by an increased amount of those proteins in plasma.

Western blot analysis of kallistatin allowed detection of two protein bands: the heavier one at 48 kDa that represents the molecular weight of the whole sequence of the protein, and a second one that may represent the molecular weight difference corresponding to a signal peptide at the N-terminal side of the protein which molecular mass corresponds to 2203 Da. AAA to non-AAA ratio was significantly different for the lighter protein band, suggesting an up-regulation of the activated form of kallistatin in AAA plasma samples. Together with carboxypeptidase B2, which also appeared to be over-expressed in AAA plasma samples compared to non-AAA (AAA to non-AAA ratio in Western blot analysis: 1.6), are proteins involved in the regulation of the kallikrein-kinin system (Figure 4).

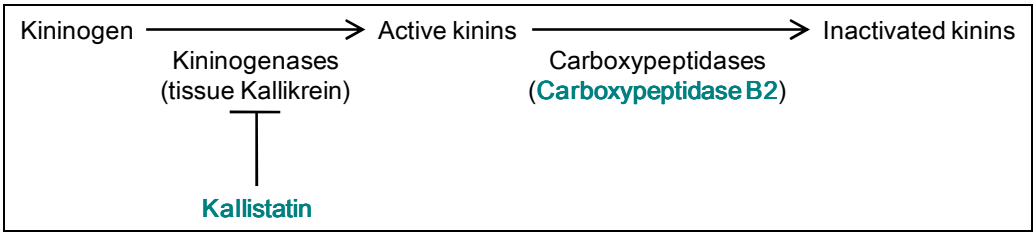


Figure 4. Schematic representation of kallikrein-kinin system regulation. Kininogen is cleaved by kininogenases, such as tissue kallikrein, to generate active kinins. Kallistatin is a specific inhibitor of tissue kallikrein. Carboxypeptidases, such as carboxypeptidase B2, can cleave C-terminal arginine or lysine residues from active kinins, to inactivate them thereby regulating their activities.

It seems that an increase of kallistatin levels may be translated by a decrease of active kinins. In the same way, an increase of carboxypeptidase B2 levels may generate an increase on kinin degradation for their subsequent inactivation. These results suggest a relationship between decreased levels of kinins and the presence of AAA. Interestingly, it has been demonstrated that kininogen deficiency promotes the development of AAA in kininogen-deficient Brown Norway rats after feeding with a high-fat (atherogenic) diet [Kaschina *et al.* 2004]. We could hypothesize that our results are in agreement with this findings because the absence of kininogen also implies the absent of active kinins. On the other hand, kallistatin has been also described as a potent vasodilator, independent of its interaction with tissue kallikrein [Chao *et al.* 1997]. Thus, increased concentration of this protein in plasma samples from AAA patients compared to non-AAA patients may help to explain the presence of AAA.

4.4. General discussion on plasma sample analysis

Plasma samples were analyzed by two different quantitative proteomic strategies: by 2D-gel based differential proteomics and by two nLC-MS/MS quantitative approaches.

Proteomic analysis of plasma samples of 8 AAA and 16 non-AAA patients by 2D-gel based techniques was performed by two different strategies: performing 2D-gels either on individual plasma samples or pooled plasma samples. When analysis was performed on individual plasma samples, bioinformatic analysis of silver-stained 2D-gels revealed no differentially expressed spots among the 882 spots detected *per* image. However, when 2D-gel corresponding to four replicates of each group of pooled plasma samples, bioinfomatic analysis showed 4 differentially expressed spots among the 1170 spots detected *per* image: 1 up-regulated and 3 down-regulated in AAA pooled plasma samples compared to non-AAA pooled plasma samples. These spots had a fold-change equal or greater than 1.5. These results suggest that pooling of plasma samples can avoid effects of interindividual biological variation, leading to the observation of differentially expressed spots that

could not be detected by analysis of individual plasma samples. Unfortunately, due to a technical problem during albumin and IgG depletion of plasma samples, that we could not solve, proteome profile of preparative gels did not correspond to proteome profile of analytical gels and differentially expressed spots could not be detected. Consequently, differentially expressed proteins corresponding to these spots were not identified by MS analysis.

Regarding nLC MS/MS analysis, plasma samples of 17 AAA and 17 non-AAA patients were analyzed by two different MS approaches. The first approach combined the use of PAcIFIC MS analysis with spectral count while the second approach combined the use of data independent MS analysis with TMT isobaric labeling. PAcIFIC MS analysis allowed protein identification with a dynamic range of 4.5E+6, that was calculated taking account of the 360 multiple hit protein identifications. Spectral count analysis of these proteins allowed selection of 15 differentially expressed proteins to be validated by Western blot analysis. The second approach combined the use of data independent MS analysis with TMT isobaric labeling. One hundred forty seven proteins, that contained, at least, two peptides for which both TMT reporter ions were detected and protein ratios could be calculated, were evaluated for differential expression levels between plasma samples from AAA and non-AAA patients. Among evaluated proteins, 7 proteins were additionally selected to be validated by Western blot analysis.

After validation of the 22 selected proteins by Western blot analysis, four proteins were found to be differentially over-expressed in AAA plasma samples compared to non-AAA plasma samples: adiponectin, extracellular superoxide dismutase, kallistatin and carboxypeptidase B2. All of these proteins are described to be potentially involved in aneurismal pathology. For instance, adiponectin in an adipokine with several properties such as anti-inflammatory, anti-atherosclerotic, and anti-diabetic properties. Thus, increased levels of adiponectin in AAA plasma samples help to explain the negative association between diabetes and AAA. Extracellular superoxide dismutase has been described to be under-expressed in AAA tissue compared to control tissue. Our results showed over-expression of extracellular superoxide dismutase in AAA plasma samples compared to non-AAA plasma samples. These results appear to be complementary and suggest an equilibrium between the amount of protein expressed in tissue and the amount of protein released into the blood stream. Finally, kallistatin and carboxypeptidase B2 are proteins involved in the regulation of the kallikrein-kinin system. Up-regulation of these proteins may be translated as a down-regulation of active kinins. The absence of kininogen (inactive precursor of kinins) has been described to develop AAA in animal models. Congruently, regulation of the kallikrein-kinin system for the suppression of kinin expression appears be consistent with the presence of AAA. Large cohort studies are needed to validate the application of these four potential biological markers for the screening of AAA in the population at

risk. That will allow early diagnosis of the disease, decreasing mortality rate of elderly population in developed countries.

Regarding the different proteomic techniques that were used, in 2D-gel based proteomics, analysis and identification of proteins are performed in two different steps, whereas in nLC-MS/MS approaches both steps are performed at the same time. The application of one or the other type of proteomic analysis is a choice that depends on the number of samples to be analysis, time available to perform the analysis and the specific biological question that needs to be answered by the study. Analysis by 2D-gel based approaches is less time consuming and allows detection of post-translational processing of proteins although it is a process much less automated than nLC-MS/MS, that may be consider more reproducible. However, a clear example of the power of 2D-gel based analysis is shown by the analysis of cell protein extracts by 2D-DIGE, in which the technical bias produced by the treatment of the samples with DNase I could be noticed just observing gel images before performance of the bioinformatic analysis. Thus, gel based and gel free proteomic approaches can be considered as complementary because of the different information that can be extracted from these types of analysis.

5. Technical contributions to the laboratory during PhD studies

This section contains a brief description of the main technical contributions that have become part of a routine way of working in the group.

5.1. MALDI-TOF MS spectra treatment

Usually, a MALDI-TOF MS spectra needs to be treated before obtaining the list of m/z values of the peaks that will be used for protein identification. Classically, this treatment is performed by adapted software and is composed of a first step of peak calibration followed by a peak detection step. DataExplorer (PerSeptive Biosystems, Framingham, MA, USA) software is used in our group to process MALDI-TOF spectra obtained after MS analysis with a Voyager DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Initially, process of peak detection was performed in two steps: baseline correction and manual peak detection. For manual peak detection, the spectra was divided into three m/z ranges (800 to 1029; 1029 to 2200; and 2200 to 3000) and a minimum intensity threshold was defined for each m/z range in order to establish when a peak was consider either as a peptide peak or a noise peak. These intensity thresholds were defined by the user, and consequently, they might be subjective. Thus, the analysis of the same spectra by two different users could give rise to two different peak lists for protein identification.

In order to optimize the treatment of MALDI-TOF MS spectra, a very simple macro was created to be implemented in DataExplorer software. This macro performs the treatment on the

MALDI spectra as follow: Firstly, baseline is corrected and made it equal to zero; secondly, noise is reduced using the default parameters of the software; then, all peaks presented in the spectra in a m/z range from 800 to 3000 are detected and m/z values are annotated without restriction; after that, a process of deisotoping is performed, in which mathematic algorithms that take into account m/z distribution of the peaks are used to select monoisotopic m/z values of peptides. Finally, from monoisotopic peaks, only those with intensity value higher than 5% of the highest peak in the spectra are selected (a maximum of 40 peaks can be selected for protein identification, the most intense ones). Using this macro, treatment of MALDI-TOF spectra became a process much more objective and reproducible.

In some cases, intensity threshold in the last step can be set at a different value than 5% or peak with m/z < 900 can be discarded (matrix peaks), depending on the quality of the mass spectra. However, one has to be critical and be able to decide from the beginning whether a spectrum is good for protein identification or not. MALDI-TOF MS is a multi-factorial process and quality of the resulting spectra is based in all steps from protein digestion and peptide extraction to matrix crystallization or the laser power that is applied.

5.2. In-gel digestion of proteins labeled with Cy3 saturation DIGE dyes

During DIGE analysis using saturation dyes, once 2D-DIGE analytical gels have been compared and differentially expressed spots have been selected, identification of proteins is performed on spots from preparative gels. Usually, to perform these preparative gels, 500 µg of protein are labeled with Cy3 saturation dye before isoelectrofocusing and electrophoresis steps. Cy3 saturation dyes are water-soluble fluorescent dyes that react with the thiol group of cysteine residues thanks to a maleimide reactive group included in their chemical structure. An additional mass of 672.9 amu is added to each protein *per* labeled cysteine. The influence of Cy3 labeling of proteins in mass spectrometry has been studied by Sitek *et al*. [Sitek *et al.* 2005]. In this study, the authors described that 96.3% of all human proteins carry at least one cysteine, whereas more than 76.3% have more than ten cysteines, and due to the very low identification of cysteine-containing peptides by both MS and MS/MS analyses, they concluded that the Cy3 labeling may suppresses ionization of the peptides or a possible metastable fragmentation led to unknown cysteines adducts of so far unknown masses.

In our case, MALDI-TOF MS spectra of spots from Cy3 labeled proteins were extremely poor in content of peaks and did not allowed protein identification using the standard digestion and extraction protocols applied in the group, that worked out fine for non-labeled proteins. In order to resolve this trouble, we focused on the digestion step, assuming that it could be possible that trypsin

does not have an optimize activity due to the voluminous and hydrophobic structures of Cy3 dyes (Figure 1) that surround proteins and work as repelling artifacts.

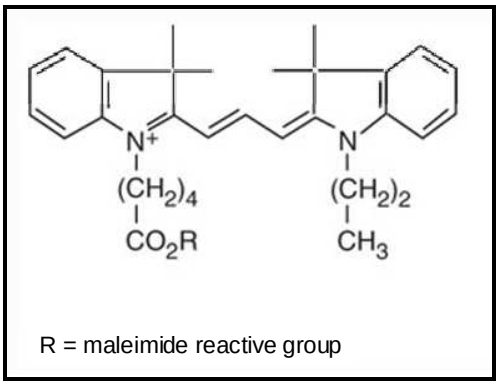


Figure 1. Chemical structure of Cy3 saturation DIGE dye

ProteaseMAX™ Surfactant, Trypsin Enhancer (Promega), is a commercial product designed to improve in-gel and in-solution protein digestion. We decided to test the utilization of this product during in-gel trypsin digestion of proteins. For that aim, a SDS-PAGE gel was performed loading 10 µg of monocyte-derived macrophage protein extract labeled with Cy3 in one lane, and 10 µg of the same protein extract without labeling in another lane (Figure 2). One random band in each lane was selected and cut into two equal pieces.

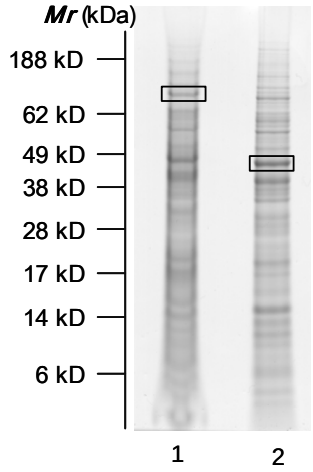


Figure 2. Coomasie stained SDS-PAGE of 10 µg of monocytes-derived macrophage protein extracts. 1: Cy3 labeled protein extract. 2: Non-labeled protein extract. The positions of molecular weight (M*r*) standards are indicated on the left. Highlighted with a black frame: bans used for in-gel trypsin digestion.

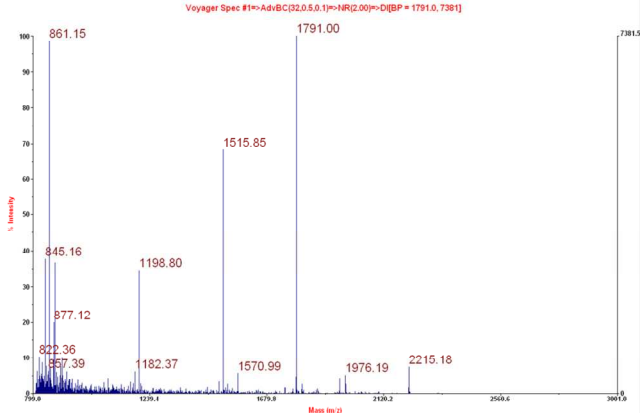
For one gel piece of each band in-gel digestion of proteins was performed classically. Briefly, before digestion, spots were washed for 15 min with 100 µL of 50 mM ammonium bicarbonate

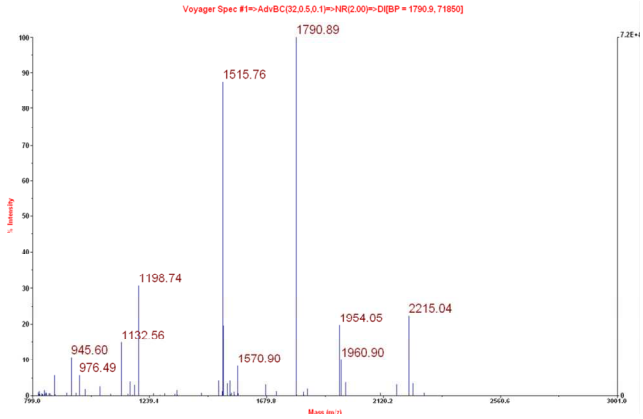
solution and then twice for 15 min with 50 mM ammonium bicarbonate/50% ACN. They were then dried after adding 100 µL of ACN for 10 min. After discarding the supernatant, tubes were left open for 10 min to complete solvent evaporation. They were then rehydrated with 12 µL of a solution containing 50 mM ammonium bicarbonate and 3 µL of 40 µg/mL Trypsin Gold (Promega) in 50 mM acetic acid. After overnight digestion at 30°C, digestion was stopped by addition of 8 µL of ACN and incubation at 37º for 15 min. Next, peptides were extracted by incubation with 130 µL of 0.2% TFA at room temperature for 30 min. The supernatant was then transferred into a new tube. Peptides were then purified, desalted with ZipTip C18 tips (Millipore Bedford, MA, USA) according to the manufacturer's protocol, and eluted with 3 µL of 0.1% TFA/50% ACN.

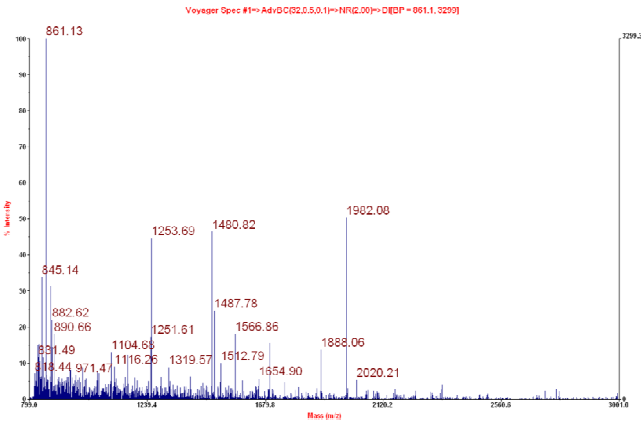
For the other gel piece of each band in-gel trypsin digestion was performed using ProteasMAX™ Surfactant, as follows: before digestion, spots were washed for 15 min with 100 µL of 50 mM ammonium bicarbonate solution and then twice for 15 min with 50 mM ammonium bicarbonate/50% ACN. They were then dried after adding 100 µL of ACN for 10 min. After discarding the supernatant, tubes were left open for 10 min to complete solvent evaporation. They were then rehydrated with 12 µL of a solution containing 0.025% ProteasMAX™ Surfactant, Trypsin Enhancer (Promega) in 50 mM ammonium bicarbonate and 3 µL of 40 µg/mL Trypsin Gold (Promega) in 50 mM acetic acid. After overnight digestion at 37°C, peptide extraction was carried out in two steps according to the manufacturer's protocol for ProteasMAX™ Surfactant, Trypsin Enhancer. First, after adding 10 µL 0.01% Surfactant solution and shaking for 10 min, we transferred the supernatant into a new tube and added 20 µL of 0.01% Surfactant, 1% TFA solution; after the tube was shaken for 10 min, the supernatant was transferred with the first peptide extraction. Peptides were then purified, desalted with ZipTip C18 tips (Millipore Bedford, MA, USA) according to the manufacturer's protocol, and eluted with 3 µL of 0.1% TFA/50% ACN.

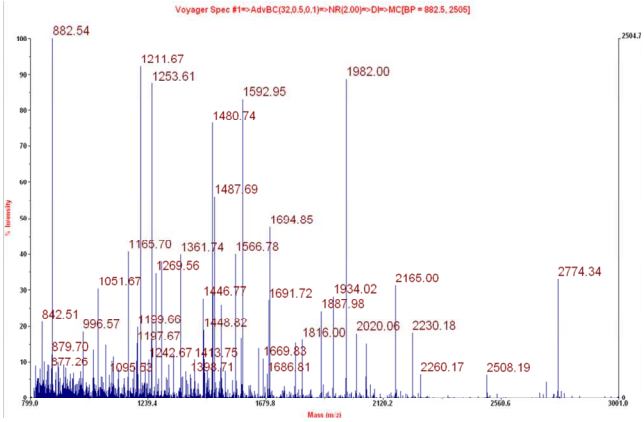
Extracted peptides of the four gel pieces were analyzed by MALDI-TOF MS. Treated spectra as well as the corresponding screen shots of protein identifications by MASCOT engine are shown in Figure 3. When trypsin digestion was performed using ProteasMAX™ Surfactant, MALDI-TOF MS spectra were more enriched in peaks than in the absence of ProteasMAX™ Surfactant for both Cy3 labeled and non-labeled protein extracts. Moreover, the number of low m/z peaks (m/z < 900) corresponding to matrix adducts was importantly decreased, and/or intensity for those peaks was lower, in spectra corresponding to ProteasMAX™ Surfactant trypsin digestions than in spectra corresponding to trypsin digestions.

A









Mascot Search Results

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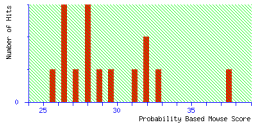
User          : a
Email         : adelina.acosta@pasteur-lille.fr
Search title  : MDM177_47_240608
Database      : NCBInr 20080616 (6589360 sequences; 2253133281 residues)
Taxonomy      : Homo sapiens (human) (205115 sequences)
Timestamp     : 25 Jun 2008 at 10:17:30 GMT
Top Score     : 38 for gi|15277503, ACTB protein [Homo sapiens]
```

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 66 are significant (p<0.05).



Mascot Search Results

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Email         : adelina.acosta@pasteur-lille.fr
Search title  : MDM177_47S_240608
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Taxonomy      : Homo sapiens (human) (205115 sequences)
Timestamp     : 25 Jun 2008 at 10:20:22 GMT
Top Score     : 109 for gi|15277503, ACTB protein [Homo sapiens]
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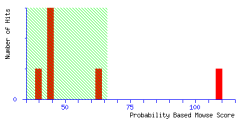
Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 66 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 10

Re-Search All | Search Unmatched

1. gi|15277503 Mass: 44228 Score: 109 Expect: 2.6e-06 Queries matched: 8
ACTB protein [Homo sapiens]

B

Mascot Search Results

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User          : a
Email         : adelina.acosta@pasteur-lille.fr
Search title  : MDM177_5T_240608
Database      : NCBInr 20080616 (6589360 sequences; 2253133281 residues)
Taxonomy      : Homo sapiens (human) (205115 sequences)
Timestamp     : 25 Jun 2008 at 10:33:03 GMT
Top Score     : 35 for gi|3153290, immunoglobulin light chain variable region [Homo sapiens]
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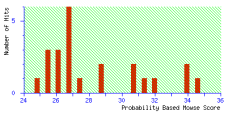
Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 66 are significant (p<0.05).



Mascot Search Results

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User          : a
Email         : adelina.acosta@pasteur-lille.fr
Search title  : MDM177_5TS_240608
Database      : NCBInr 20080616 (6589360 sequences; 2253133281 residues)
Taxonomy      : Homo sapiens (human) (205115 sequences)
Timestamp     : 25 Jun 2008 at 10:34:41 GMT
Top Score     : 98 for gi|6470150, BiP protein [Homo sapiens]
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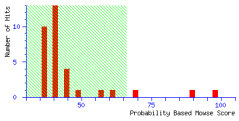
Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 66 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 10

Re-Search All | Search Unmatched

1. gi|6470150 Mass: 72232 Score: 98 Expect: 3.5e-05 Queries matched: 22
BiP protein [Homo sapiens]
gi|16307237 Mass: 73633 Score: 83 Expect: 0.0011 Queries matched: 22
heat shock 70kDa protein 5 [Homo sapiens]

Figure 3. MS identification of digested bands of the SDS-PAGE performed with 10 µg of macrophage protein extract. A: Cy3 labeled proteins. MALDI-TOF MS spectra and the corresponding MASCOT identification of trypsin digested proteins are shown in the upper part, and MALDI-TOF MS spectra and the corresponding MASCOT identification of ProteasMAX™ Surfactant assisted trypsin digestion are shown in the lower part. B: Non-labeled proteins. MALDI-TOF MS spectra and the corresponding MASCOT identification of trypsin digested proteins are shown in the upper part, and MALDI-TOF MS spectra and the corresponding MASCOT identification of ProteasMAX™ Surfactant assisted trypsin digestion are shown in the lower part.

Regarding protein identification of digested bands, trypsin digestion of Cy3 labeled proteins did not give rise to any significant hit. The hit with the highest value of mowse based score, which is 38, was for β-actin. However, ProteasMAX™ Surfactant trypsin digestion allowed identification for β-actin with a significant mowse based score of 109. In the case of non-labeled proteins, results were similar. Trypsin digestion of proteins did not get any significant identification whereas ProteasMAX™ Surfactant trypsin digestion resulted in the identification of a few proteins with significant mowse based scores. In the case of a real study, distinction between protein candidates can be achieved by MS/MS analysis of digested peptides.

These results largely demonstrated the efficacy of ProteaseMAX™ Surfactant improving trypsin in-gel digestion of both non-labeled and Cy3 labeled proteins. This product helps trypsin to have access to proteins and increases the yield of peptide extraction from the gel matrix. Thus, protein digestion gives rise to a greater number of peptides, which m/z values will ideally allow a better identification of proteins of interest.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

V. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In this PhD work we have used differential proteomic techniques in order to analyze and compare different kinds of samples from patients presenting an AAA to unaffected patients. These analyses were performed in order to identify potential biological markers that would ensure early diagnosis and subsequent early treatment of this dramatic disease, and to provide a better understanding of the physiopathological mechanisms behind AAA formation and evolution.

In 2002, a clinical protocol was established for the recruitment of patients undergoing coronary bypass surgery. This clinical protocol allowed, over a 4 year period, the obtention of plasma samples and cells, which were cultured *in vitro* for further protein extraction. Macrophages were derived from circulating monocytes and SMC were isolated from a residual segment of internal mammary artery used for the bypass, a region located far from the abdominal aorta. Hence, it could be criticize the fact that cell types that were analyzed did not came directly from aneurismal tissue. However, it is important to notice that echography examination to check the presence of AAA in recruited patient was performed after the bypass surgery. Moreover, since for almost all patient, AAA did not have the recommended size to be surgically operated, the obtention of aneurysmal tissue was limited. If we had to design a new clinical protocol for the discovery of AAA biomarkers, probably samples from patients undergoing AAA repair were required. Thus, since aneurysmal pathology could be consider in an advance stage, larger differences might be found between protein profile of AAA and non-AAA samples. Moreover, that would allow the analysis of cell types that come directly from the affected tissue.

Protein samples extracted from biological samples obtained from recruited patients were expected to be analyzed by 2D-gel based techniques when all patients had been enrolled in the clinical protocol and all protein samples were available. Our group has a large experience in the application of 2D-gel based techniques for the analysis of protein samples. Long term clinical studies, in which biological samples of patients are collected, require a strong rigorous character during cell sampling, cell culture, protein extraction and protein storage, steps that must be performed the same way throughout the study. In the present study we have applied the guideline for clinical proteomic studies described by Mischack et al. [Mischak *et al.* 2007].

All steps from cell sampling to protein storage have been carefully applied the same way over the 4 years but unfortunately, the use of saturation DIGE, together with SameSpots software, allowed the detection an unexpected technical bias, that modified proteome profile, during the application of DNase I treatment on the samples for the elimination of nucleic acids. Proteins affected by the technical bias that were those involved in cell motion, actin cytoskeleton

reorganization, and apoptosis. The use of DNase I is demonstrated to be not appropriated for the removal of nucleic acids from cell protein extracts that have been collected during long term studies.

Despite the power of DIGE analysis for detection of differentially expressed proteins, MS analysis of interesting spots was limited by the labeling of the proteins with Cy3 DIGE saturation dyes. Classical trypsin digestion did not allow protein identification of labeled spots. Most of the time, obtained spectra were “empty” and when few peaks were present, they were not enough to allow protein identification. Only with the application of ProteaseMAX™ Surfactant during trypsin digestion of spots, proteins could be identified. ProteaseMAX™ Surfactant clearly showed an important improvement in both digestion efficacy and peptide extraction recovery.

Unfortunately, comparison between AAA and non-AAA cell samples did not get any concluding result. This may be due to the presence of the technical bias. However, because all patients underwent bypass surgery, we also hypothesize that the strong influence of the atherosclerotic phenotype did not allow detection of differentially expressed proteins specifically involved in aneurismal disease.

Plasma samples were firstly analyzed by 2D-gel based differential proteomics applying two different strategies: performing 2D-gels either on individual plasma samples or pooled plasma samples. When analysis was performed on individual plasma samples, bioinformatic analysis of silver-stained 2D-gels revealed no differentially expressed spots. However, during the analysis of pooled plasma samples, 4 spots were found to be differentially expressed between AAA and non-AAA plasma samples, suggesting that pooling of plasma samples can avoid effects of interindividual biological variation. Unfortunately, differentially expressed proteins corresponding to these spots could not be identified by MS analysis due to a technical problem during albumin and IgG depletion of plasma samples. We obtained preparative 2D-gels with proteome profiles that did not correspond to proteome profile of analytical gels. In order to solve this problem, pooled plasma samples of AAA and non-AAA patients were analyzed by a label-free MS approach and a label-based MS approach in collaboration with the laboratory of Pr Goodlett in Seattle (USA). Both methodologies that were applied used data independent MS analysis. Importantly, without any previous fractionation of the sample other than depletion of the 14 most abundant proteins, this method allowed protein identification over a concentration dynamic range of 4.5E+6. Label free quantification was performed by spectral count analysis, allowing selection of 15 differentially expressed proteins to be validated by Western blot analysis. Labeling approach was performed using TMT isobaric tags and allowed additional selection of 7 proteins for further validation by Western blot analysis.

Western blot analysis was performed on non depleted plasma. Of the 22 selected proteins, only 8 could be detected, suggesting that protein concentration enrichment by depletion of the 14 most abundant proteins in plasma was a critical step that allowed an increase of sensitivity during MS analysis. Western blot analysis on depleted plasma samples will be performed in order to validate differential protein expression of the other 14 proteins that appeared to be differentially expressed by MS quantitative analysis. Interestingly, 4 proteins were validated by Western blot as being differentially over-expressed in AAA plasma samples compared to non-AAA plasma samples: adiponectin, extracellular superoxide dismutase, kallistatin and carboxypeptidase B2. All of these proteins are described to be possibly involved in aneurismal pathology. For instance, adiponectin is an adipokine with several properties such as anti-inflammatory, anti-atherosclerotic, and anti-diabetic properties. Thus, increased levels of adiponectin in AAA plasma samples may help to explain the negative association between diabetes and AAA. Extracellular superoxide dismutase has been described to be under-expressed in AAA tissue compared to control tissue. Our results showed over-expression of extracellular superoxide dismutase in AAA plasma samples compared to non-AAA plasma samples. These results appear to be complementary and suggest an equilibrium between the amount of protein expressed in tissue and the amount of protein released into the blood stream. Finally, kallistatin and carboxypeptidase B2 are proteins involved in the regulation of the kallikrein-kinin system. Up-regulation of these proteins may be translated as a down-regulation of active kinins. The absence of kininogen (inactive precursor of kinins) has been described to develop AAA in animal models. Congruently, regulation of the kallikrein-kinin system for the suppression of kinin expression appears be consistent with the presence of AAA.

None of these four proteins have been previously described as potential biomarkers for AAA diagnosis [Hellenthal *et al.* 2009a; Hellenthal *et al.* 2009b; Urbonavicius *et al.* 2008; Golledge *et al.* 2008b]. Indeed, most of the studies assess potential biomarkers that were *a priori* selected because of their role in biological mechanisms involved specifically in AAA or generally in cardiovascular pathology. Thus, several epidemiological studies have been performed, resulting in non concluding results. To our knowledge, the present study assessed for the first time the application of differential proteomic techniques for the discovery of AAA biological markers, comparing plasma proteomic profiles of affected patients to those of unaffected patients. One of the main advantages of proteomic analysis is the absence of any *a priori* hypothesis, so these techniques are able to lead to the identification of new biomarker candidates, offering new alternatives on biomarker research. The present study is in the framework of the first step of biomarker development: the discovery phase. Further studies on large multicenter cohorts are warranted to validate these results.

Perspectives for this study will focus on the use of alternative techniques such as laser microdissection for the analysis of cell samples directly isolated from aneurysmal tissue. This new strategy avoids the difficulties found during the present study. First, aneurysmal biopsies are easier to store because they only need to be frozen quickly in liquid nitrogen from the moment they are obtained at the hospital. Second, cell sampling does not need to be performed in several steps at different time during the recruitment of patients. Once all tissues are collected, cell samples can be obtained for protein extraction at the same time. Third, the histological and morphological changes of the aortic wall due to degradation of the extracellular matrix allow a better comprehension of the pathology than the study of cultured cells. Interestingly, the question of which type of immune response is involved in AAA can be also addressed by this strategy. Two different phenotypes of macrophages have been described according to Th1 and Th2 immune responses: macrophages M1 and macrophages M2 respectively [Martinez *et al.* 2008]. Preliminary results in our group show that both types of macrophages are present in AAA tissue. The study of the localization as well as the different proteome profiles of these cell types will help to the better understanding of the role of inflammation and inflammatory cells during AAA formation and evolution.

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VI. REFERENCES

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APPENDIX

VII. APPENDIX

This section contains a brief scientific review written by our group, which summarizes the importance of proteomic analysis in cardiovascular diseases.

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PROTEOMIC ANALYSIS IN CARDIOVASCULAR DISEASES

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SUMMARY

1. Cardiovascular diseases are a major cause of morbidity and mortality in western countries. The molecular mechanisms responsible for heart dysfunction are still largely unknown, except in cases of genetic defects or alteration of genes and proteins.

2. The publication of genome sequences from humans and other species has demonstrated the complexity of biology, including the finding that one gene does not encode for only one protein but for several, due to mRNA splicing and post-translational modifications.

3. Proteomic analysis can provide an overall understanding of changes in the levels of protein expression. Differential proteomics is a powerful tool for improving our understanding of integrated biochemical responses. The main techniques used are two-dimensional electrophoresis (2D-gel) and Surface-Enhanced Laser Desorption/Ionization Time of Flight (SELDI-TOF) to separate proteins associated with mass spectrometry. Bioinformatic tools make it possible to compare protein profiles obtained from diverse biological samples.

4. The combination of these approaches has proved to be particularly interesting for studying cardiovascular diseases and thereby improving our understanding of the mechanisms involved and identifying new biochemical factors and biomarkers involved in these diseases.

Key words: biomarkers, cardiovascular cells, cardiovascular tissues, mass spectrometry, plasma, SELDI-TOF, two-dimensional electrophoresis.

INTRODUCTION

The complete sequencing of the human genome in 2001[1,2] showed that fewer than 3000 genes encode more than a million proteins. One gene can encode not only a single protein but several proteins, depending on the number of transcriptional (mRNA splicing) and post-translational (glycosylation) modifications. Currently the Swiss-Prot protein database contains 276 256 annotated proteins in UniProtKB/Swiss-Prot (release 54.0 of 4 July 2007) and 4 672 908 proteins translated from the European Molecular Biology Laboratory (EMBL) nucleotide sequence in UniProtKB/TrEMBL (release 37.0 of 4 July 2007). These data suggest that the genome does not reflect the organism's functional complexity, which is inversely correlated with its biological complexity (Fig. 1).

Classical strategies have measured mRNA transcripts or proteins according to a *priori* hypothesis based on literature reviews. Because of the need for global approaches without any a *priori* hypothesis, techniques known as 'OMICS' have been developed for use in analysing genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics). These techniques should make it possible to elucidate the functional role of several genes or gene products and thus better understand phenotypes linked to various types of disease status. The importance of measuring proteins has become clear, as mRNA transcripts cannot be directly correlated with protein expression[3] and post-translational modifications, such as phosphorylation and glycosylation are known to produce several proteins with different functions from a single gene. Table 1 summarizes the most common of the more than 200 post-translational modifications that have so far been described. These modifications can be seen only at the protein level and they play a role in protein abundance and in cellular localization. The notion of cartography for protein level expression is an old one, dating back to the publication of a technique to separate proteins simultaneously in 2D electrophoresis gel.[4] The term 'proteome', however, was first used in 1994 by Wilkins and defined as all proteins expressed by a genome, cell or tissue.

Proteomics is the analysis of the proteome to characterize qualitatively and quantitatively all of the proteins presented in a biological sample obtained in defined conditions. Differential proteomics is the comparison of protein profiles from various samples obtained in different conditions to identify proteins differentially expressed without any a *priori* hypothesis.

Proteomic analysis strategies

Several strategies, summarized in Fig. 2, are often used in differential proteomic analysis. Samples can be analysed with or without preliminary treatment. In some conditions, the presence of major proteins detection can make it difficult to detect rare proteins or peptides. These major proteins can be either discarded or equalized[5] to enrich the samples in minor proteins.

Proteomic analysis can be divided in four main steps: (i) processing samples, which has been described above; (ii) separation of proteins and peptides; (iii) detection and quantification of

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List of abbreviations:

2 DE	bi-dimensional electrophoresis	*pI*	isoelectric point
IPG	immobilized pH gradient	SELDI-TOF	Surface-Enhanced Laser Desorption/Ionization Time of Flight
MS	mass spectrometry	SMC	smooth muscle cells

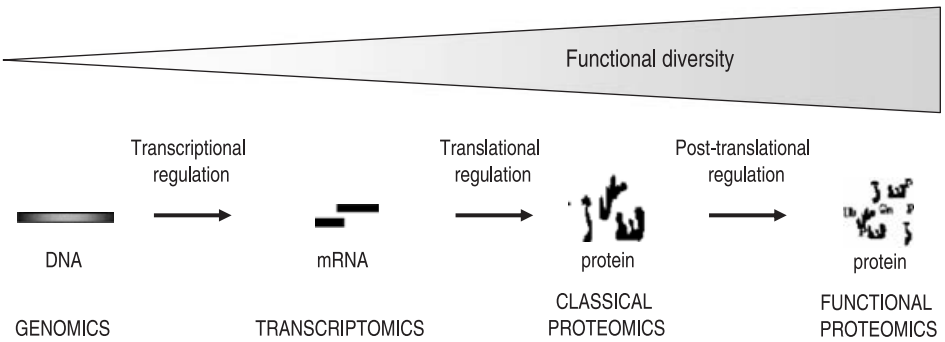

Fig. 1 Contribution of proteomic analysis to studying the functional diversity of genome expression. Inverse correlation between the complexity of the biology and the technology. Three 'OMICS' are represented: genomics for DNA analysis, transcriptomics for mRNA analysis and proteomics for studying the protein levels (classic proteomics) and their function due to post-translational modifications and protein–protein interactions (functional proteomics).

Table 1 Examples of post-translational modifications

Post-translational modification	Modified amino acid	Characteristic and function
Phosphorylation	Tyr, Ser, Thr	Reversible, activation/inactivation of protein activity, modulation of protein–protein interaction, signalling pathways
Acetylation	N-term Lys	Protein stability, N-terminal protection, regulation of protein–DNA interaction
Methylation	Lys	Gene expression regulation
Acylation		Cellular localization, membrane anchor
Myristoylation	N-term Gly	
S-acylation	Cys, Ser	
Glycosylation		Secreted proteins, signalling pathways, cell/cell recognition
N-linked	Asn	
O-linked	Ser, Thr	
O-GlcNAc	Cys, Ser	Reversible, cellular regulation
Hydroxyproline	Pro	Protein stability, protein/ligand interaction
Sulphatation	Tyr	Modulation of protein/protein and protein/ligand interactions
Disulphide bonds	Cys	Intra- and extra-molecular interactions, protein stability
Ubiquityinylation	Lys	Sign of protein degradation
Nitrosylation	Tyr	Oxidative damage

proteins and peptides; and (iv) purification and identification of the corresponding proteins by mass spectrometry (MS) and bioinformatic analysis.

Proteins can be separated either by electrophoresis (mono- or two-dimensional) or directly by MS techniques such as Surface-Enhanced Laser Desorption/Ionization Time of Flight (SELDI-TOF) or liquid chromatography-MS. For direct MS analysis, quantification relies on the relative intensity of peaks. Bioinformatic tools make it possible to target the spots or peaks of interest that should be identified. Spots are directly cut into the 2D gel, and for SELDI-TOF purified peaks are cut into 1D gel, for trypsin digestion and MS analysis. Other differential proteomics strategies allow direct quantification of peptides by MS (ICAT: isotope-coded affinity tag) or MS (–MS) (Isobaric Tagging Reagents for Quantitative Proteomic Analysis (iTRAQ)) after labelling, trypsin digestion and MS (–MS) analysis. This multiplex approach mixes many samples after labelling with different tags that allow them to be differentiated in MS analysis or 2D-differential gel electrophoresis (DIGE). The latter uses protein labelling of each sample by a specific fluorophore before mixing and electrophoresis. In most cases, MS that combines peptide mapping and MS-MS data finally identifies the selected proteins.

Two-dimensional gel electrophoresis

Two-dimensional electrophoresis (2DE) is a two-step technique that was developed 30 years ago[4] that makes it possible to separate proteins: (i) by their isoelectric point (*pI*), a step called isoelectro-focusing and (ii) by their molecular weight, using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Reliable proteomic analysis requires a reproducible technology of protein separation that uses immobilized pH gradient (IPG), to improve reproducibility and resolution.[6]

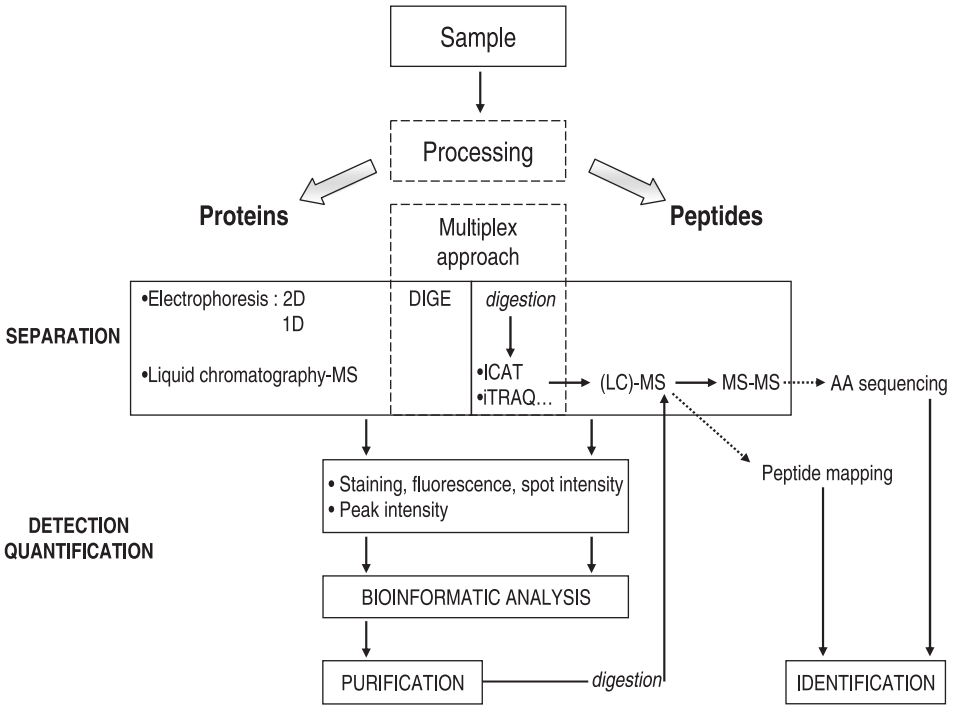

Fig. 2 Schematic representation of four steps in the proteomic analysis strategies: (i) processing samples; (ii) separating proteins and peptides; (iii) detecting and quantifying protein and peptides; and (iv) purifying and identifying corresponding proteins by mass spectrometry (MS).

After 2DE, the proteins can be visualized with either universal or specific stains. Stain properties that should be considered are sensitivity, linear dynamic range, reproducibility and compatibility for protein identification by MS. Standard universal stains are silver nitrate, coomassie blue and more recently fluorescent probes (SYPRO Ruby, FLAMINGO). Specific stains allow the visualization of, for example, phosphorylated proteins (ProQDiamond). This staining technique provides quantitative screening to analyse essentially hydrophilic proteins in a molecular mass range between 15 and 100 kDa and a pH range of 3–8. Protein detection then uses non-specific labelling such as coomassie blue, silver nitrate or fluorescent probes. Quantification is carried out directly by densitometry using an appropriate scanner and assumes that spot intensity is directly proportional to spot abundance.

The development of 2D bioinformatic software (Progenesis Samespots, Platinium, etc.) allows the detection of specific polypeptidic spots and their intensities as well as a comparative analysis of several 2D gels. These statistical tools make it possible to select spots whose intensity is modulated between several experimental conditions.

SELDI-TOF analysis

This technology has some advantages over 2DE for proteins that are hydrophobic, very basic or of low molecular mass (< 20 kDa).[7] The key elements are ProteinChips arrays of various chemical (anionic (Q10) or cationic (CM10)), hydrophobic (H50) and hydrophilic (NP20) surfaces that can divide the proteome by capturing proteins with affinity for the surface before the direct MS analysis. Spectra obtained with a ProteinChip system time-of-flight mass spectrometer can be analysed by three types of software (Biomarker Wizard, Biomarker Pattern Software and Ciphergen Express). The intensity of peaks obtained in the spectra under different experimental conditions are compared to find potential markers.

DISCUSSION

Samples

The first question is what would be the most appropriate biological samples for proteomic analysis of cardiovascular diseases – tissue, cell or simply plasma or serum? The most informative tissues come from the ventricle, atrium, aorta, artery and vessels. Their disadvantage is that they are composed of several cell types and the data obtained will concern the major proteins from the main cell type. Working at the cellular level would be more informative, but what type of cells would be best: cardiomyocytes, endothelial cells, fibroblasts, smooth muscle cells (SMC) or macrophages?

Human heart tissue is not easily accessible for routine screening. Plasma and serum, on the other hand, are readily accessible for use in screening for biomarkers and are thought to contain proteins secreted by or released from tissue. Plasma proteins are an important part of the proteome and tissue-derived proteins represent a significant fraction of it.[8] Twenty major proteins account for 99% of the plasma proteome and may thus mask minor proteins. To visualize minor proteins, we will use prefractionation techniques, the elimination of major proteins by specific antibodies or the equalization of biological samples to enrich the abundance of rare proteins in the sample.[5] The final question is: is it better to analyse serum or plasma? Serum is obtained after coagulation by a process involving many proteases that play a role in thrombus formation. SELDI-TOF analyses have shown that plasma and serum profiles differ significantly, with serum having many more peaks of low molecular mass.[9] Additional information will come from serum analysis, which reflects both protease activity and the activation status of circulating leucocytes. Accurate proteomic analysis of plasma and serum requires that the samples be processed very quickly and stored according to standard procedures.[10]

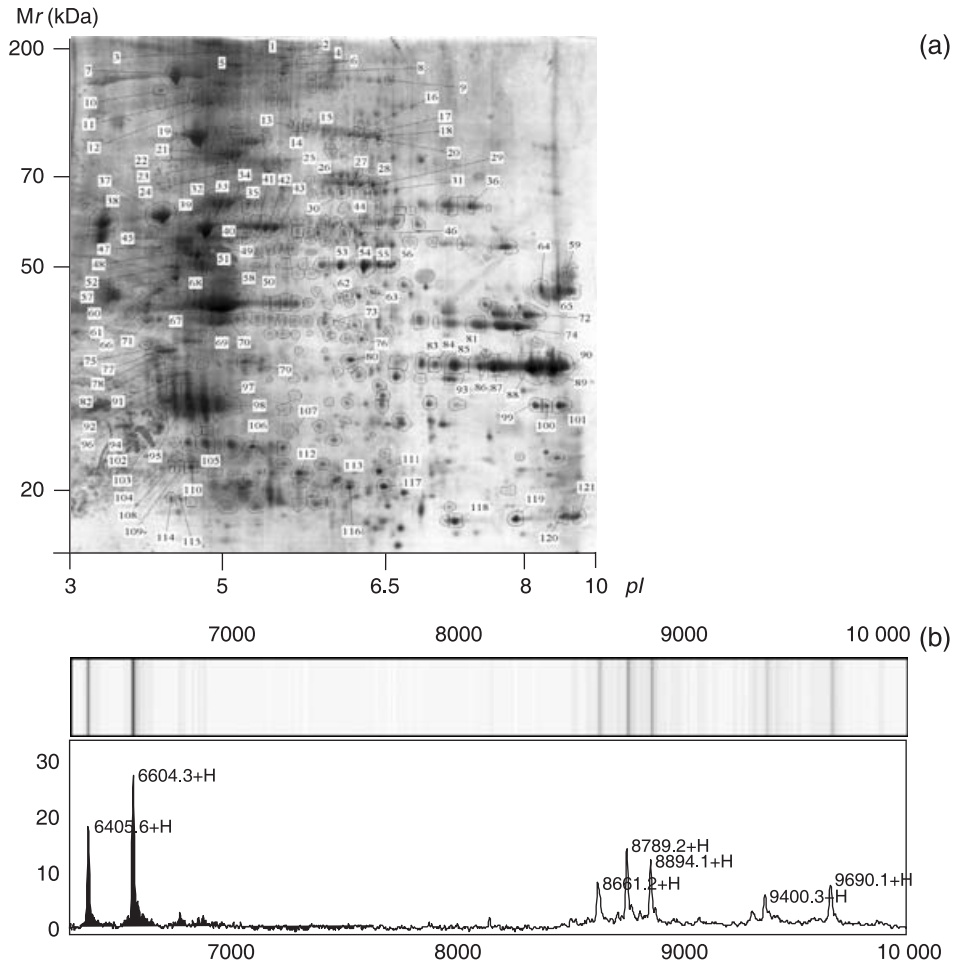

Fig. 3 Data obtained from proteomic analysis. (a) Silver-stained two-dimensional electrophoresis (2DE) gel of intracellular proteins from human smooth muscle cells (SMC). Spots surrounded were detected by bioinformatic analysis and spots numbered were identified by MS. (b) Surface-Enhanced Laser Desorption/Ionization Time of Flight (SELDI-TOF) protein spectra of plasma from heart failure patient. The upper panel presents intensity of peaks detected on gel format and the bottom panel, a spectra indicating each peak detected with the *m/z*-value.

Proteomic analysis strategies

Two examples of proteomic data obtained by 2D electrophoresis and SELDI-TOF are presented. The 2D map of SMCs (Fig. 3a) shows an average of 1500 spots detected by adequate 2D software after gel digitalization. Acid and basic proteins in Mr range of 15–200 kDa have been detected and identified by MS, carried out in accordance with recent guidelines.[11] In the past 5 years, several 2D maps related to cardiology have been published, based on endothelial cells,[12] SMCs,[13] and macrophages.[14] Several databases of 2D gels of heart tissue have also been reported.[15] Figure 3(b) presents SELDI-TOF plasma profiles of patients with heart failure, by gel format and spectra. This technique detects protein mass, intensity of peaks and quantified intensity of peaks. A recent publication used this technology to identify sTWEAK as a possible biomarker of atherosclerosis.[16]

CONCLUSION

Despite their difficulties, proteomic studies provide an opportunity to bridge the gap between molecular and systems biology, a gap that must be closed to address the many cardiovascular diseases. The development of tools for proteomic analysis opens up new possibilities for the study of the proteome and of post-translational modifications, such as phosphorylation or O-GlcNAc.[17] These studies should help to identify new targets and develop new diagnostic and therapeutic approaches for cardiovascular diseases.

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Abstract

Abdominal aortic aneurysm (AAA) is a vascular pathology characterized by an increase of aorta diameter to at least 1.5 times the diameter of reference and a loss of parallelism of the aortic wall at the infrarenal region. AAA primarily affects elderly males with a prevalence of 5%. Its rupture is responsible for 1-4% of the total mortality in males older than 65 years. In case of rupture the mortality is up to 70-95%. Thus, AAA comes up as one of the leading causes of death in industrialized countries with aging populations. Age, male gender, tobacco consumption, genetic susceptibility and the presence of another atherosclerotic localization are known clinical risk factors for the development of AAA. Rupture can be prevented by vascular surgery which decreases mortality in AAA patients. Nevertheless, most AAA patients are asymptomatic and the majority not diagnosed prior to rupture.

In this PhD work we have used differential proteomic techniques in order to analyze and compare human blood samples (plasma) and cells (smooth muscle cells and monocyte-differentiated macrophages) from patients presenting an AAA to unaffected patients. These analyses were performed with the aim of the following objectives:

a) To identify and evaluate potential biological markers for AAA screening, that will ensure early diagnosis and subsequent early treatment of this fatal disease.

b) To provide a better understanding of the physiopathological mechanisms involved in the evolution of AAA through the interpretation of the results in differential proteomic analysis.

Regarding cell proteomic analysis, the use of saturation DIGE, together with SameSpots software, allowed the identification of 2 different protein profile in both cell types independently on the disease status of the patient. These two different proteome are the result of a technical bias during the application of DNase I treatment on the samples for the elimination of nucleic acids. We showed that DNase I treatment produced changes in proteome profiles of these cell types. The proteins mainly affected by this treatment were those involved in cell motion, actin cytoskeleton reorganization, and apoptosis. Due to the presence of this technical bias, comparison between AAA and non-AAA cell samples did not get any concluding result.

Plasma proteome analysis was performed in collaboration with the laboratory of Pr. Goodlett, in Seattle (USA). Two different mass spectrometry based approaches were applied to compare plasma samples of AAA to plasma samples of non-AAA patients. The first approach combined the use of PAcIFIC MS analysis with spectral count while the second approach combined the use of data independent MS analysis with TMT isobaric labeling. After validation by Western blot analysis, four proteins were found to be differentially expressed between plasma samples of both groups of patients: adiponectin, extracellular superoxide dismutase, kallistatin and carboxypeptidase B2. These proteins are involved in aneurismal pathology and may be potential biological markers for AAA diagnosis. Large cohort studies are needed to validate their application for the screening of AAA in the population at risk. That will allow early detection of the disease and decrease of mortality on elderly population in developed countries.

Résumé

L'anévrysme de l'aorte abdominale (AAA) est une pathologie vasculaire caractérisée par une augmentation du diamètre de l'aorte de plus de 1, 5 fois le diamètre de référence et une perte du parallélisme de la paroi au niveau infra rénal. Les AAA affectent de manière prépondérante les homes âgés avec une prévalence de 5%. La rupture d 'un AAA est responsable de 1à 4% de la mortalité chez les hommes de plus de 65 ans. Dans le cas d'une rupture, la mortalité est supérieure à 70-95%. C'est pourquoi les AAA sont une des causes majeures de mortalité dans les pays industrialisés avec une population vieillissante. L'age, le sexe masculin, la consommation de tabac, la susceptibilité génétique et la présence d'une autre localisation d'athérosclérose sont des facteurs de risque connus pour le développement d'un AAA. La rupture peut être prévenue par une chirurgie vasculaire qui permet de diminuer la mortalité chez les patients atteints d'AAA. Néanmoins, la plupart des patients atteints d'AAA sont asymptomatiques et la majorité ne sont pas diagnostiqués avant la rupture.

Au cours de ce travail de thèse, nous avons utilisé des techniques de protéomique différentielle dans le but d'analyser et comparer des échantillons provenant de plasma et de cellules (cellules musculaires lisses, monocytes différenciés en macrophages) de patients présentant ou non un AAA. Ces analyses ont été réalisé avec comme but les objectifs suivants :

a) Identifier et évaluer les marqueurs biologiques potentiels pour un dépistage des AAA, qui permettrait un diagnostique précoce et donc un traitement précoce de cette pathologie.

b) De permettre une meilleure connaissance des mécanismes physiopathologiques impliqués dans l'évolution des AAA par le biais des résultats obtenus lors de l'analyse protéomique différentielle.

En ce qui concerne l'analyse protéomique des cellules, l'utilisation de la technique DIGE par saturation associée avec l'utilisation du logiciel SameSpots, a permis l'identification de 2 profils protéiques différents dans l'ensemble des types cellulaires étudiés, indépendamment du statut du patient. Ces deux protéomes différents sont le résultat d'un biais technique du à l'utilisation d'un traitement à la DNase I des échantillons dans le but d'éliminer les acides nucléiques présents. Nous avons montré que le traitement à la DNase I produisait des changements de profil protéique de ces deux types cellulaires. Les protéines principalement affectées par ce traitement sont celles impliquées dans le mouvement cellulaire, la réorganisation du cytosquelette d'actine, et l'apoptose. Du fait de la présence de ce biais technique, la comparaison entre les échantillons de cellules obtenues de patients présentant ou non un AAA n'a pas permis de résultats concluants.

L'analyse protéomique du plasma a été effectué en collaboration avec le laboratoire du Pr Goodlett à Seattle (USA). Deux approches différentes de spectrométrie de masse ont été utilisées pour comparer les échantillons plasmatiques de patients présentant ou non un AAA. La première approche combine l'utilisation de l'analyse MS PAcIFIC avec le comptage spectral et la seconde approche combine l'analyse des données MS de manière indépendante avec un marquage TMT isobarique. Après validation par western blot, quatre protéines ont été validées comme différentiellement exprimées dans le plasma de patients présentant ou non un AAA : l'adiponectine, la superoxide dismutate extracellulaire, la kallistatine et la carboxipeptidase B2. Ces protéines sont impliquées dans la pathologie anévrysmale et peuvent être des potentiels marqueurs biologiques pour le diagnostique de AAA. Des études dans de grandes cohortes seront nécessaires pour valider leur utilisation pour le dépistage des AAA dans la population à risque. Cela permettrait une détection précoce de la maladie et une diminution de la mortalité de la population âgée dans les pays industrialisés.