Review ~~a~~Article

With or without you ~~-~~— Proteomics with or without major plasma/serum proteins

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Abstract

The first sections of this review compile and discuss strategies and protocols for managing plasma/serum as a source of biomarkers relevant to human disease. In many such cases, depletion of abundant protein(s) is a crucial preliminary step to the procedure; specific conceptual and technical approaches, however, make it possible to effectively use to this purpose whole plasma/serum. The final sections focus instead on the complexity associated with each of the major serum/plasma proteins in terms of both, multiple molecular structures (existence of a number of protein species) and of multiple molecular functions (behavior as multifunctional/multitasking/moonlighting proteins). Reviewing evidence in these and some related fields (regulation of the synthetic pattern by proteins and non-protein compounds and its connection with health and disease) prompts the suggestion/recommendation that information on the abundant components of plasma/serum proteome is routinely obtained and processed/mined as a valuable contribution to the characterization of any non-physiological condition and to the understanding of its mechanisms and of its implications/sequels.

Keywords: Serum proteins; Depletion; Enrichment; Immunodepletion; Protein species

~~1.~~1 Forewords: powers of 10

There are some questions the seniors of us have answered tens of times when advising younger researchers at their first experiences with electrophoresis and proteomics. One of them is *how* to remove albumi from plasma/serum and other biological fluids before analysis. We challenge that this question is in fact the most appropriate. We maintain, first, that all major proteins in those samples are at issue; then, that a prope question should be, instead, *when* to remove them and *when* do not. In the following, we are going to present our point of view ~~-~~— which we know is not shared by the majority of the scientific community. Most of th information will focus on human specimens although similar concepts and procedures apply to plasma/serum from laboratory and farm animals as well.

Clinical biochemistry has long recognized that the concentration in blood of tissue components increases as a result of tissue damage. The recognition of tissue-specific isoforms, either as sequence variants o as heteromultimeric assembly variants, has been the basis for discriminating tissue origin. The difference in subcellular derivation (plasma membrane, cytoplasm, mitochondria) has been associated wit nature/severity/duration of the noxa. The typical intracellular proteins currently quantitated in blood by the clinical biochemistry laboratory are medium-abundance enzymes, assayed through their catalytic activity, an high-abundance structural proteins, identified through their immunological reactivity.

Either innovative or more effective disease markers should be as sharply as possible both *tissue- and disease-specific*. Proteomics studies have demonstrated that some major biological limitations exist in thes directions. The thorough investigation about the tissue distribution of gene products in different organs and cell types carried out under 'The Human Protein Atlas' project ~~-~~— which participates in the overall 'Th

Human Proteome' endeavor ~~-~~— has demonstrated that *differences in proteome composition among cell types are more of a quantitative than of a qualitative nature*. Absolute tissue specificity has been demonstrated only for small percentage of proteins, the most obvious of which had been known for decades. Even more disturbing for its practical, and more intriguing for its biological implications, is the finding that, under conditions o cellular stress, the *changes in proteome composition hardly depend on the nature of the stress*.

How does the circulating concentration of current and perspective disease markers compare with that of other proteins in plasma/serum?

Fig. 1 ~~-~~— redrawn from [1] ~~-~~— provides an overview on the quantitative relationships among the various classes of proteins within plasma/serum proteome. The overall dynamic range for their concentration exceeds 10^{10}-fold. The range associated with proteins secreted continuously, if at a varying rate, in blood (*e.g.* binding/transport proteins, protease inhibitors, coagulation factors, immunoglobulins) is wider than 10^{5}-fold, that for proteins discontinuously secreted in blood (as long-range extracellular effectors e.g. hormones and cytokines) is narrower than 10^{2}-fold. The remaining > 10^{3}-fold corresponds to the concentration range for proteins not targeted for secretion but leaking from tissues either as intact molecules or as proteolytic fragments.

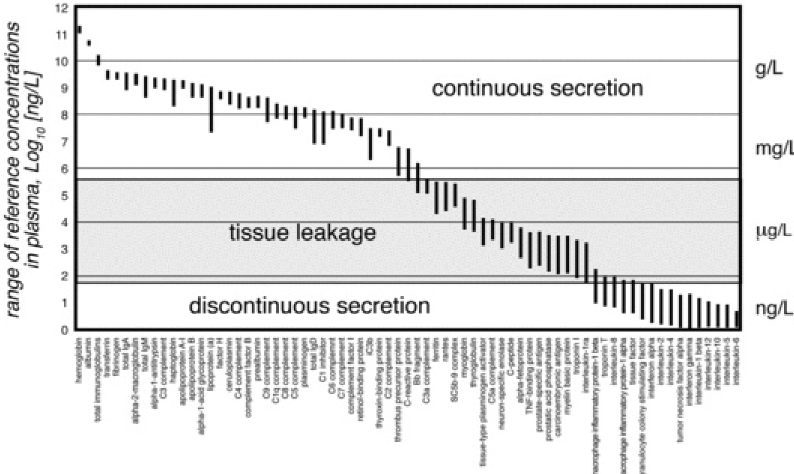

Fig. 1 A chart of the circulating concentrations of different classes of proteins found in plasma/serum. (Redrawn from [1]).

alt-text: Fig. 1

Proteins leaking from tissues would become major components of a sample only after the removal of hepatocyte and plasma cell secretion products, which are 10^{3}–10^{6} times more abundant. The subtractio of albumin alone results in the removal of 50% of the total proteins: while outstanding *per se*, such an achievement is still inadequate to meet the requirement for trace component enrichment. A suitable protocol usin the depletion approach demands a much more extensive cut of (all) major proteins. One way to meet this requirement is through the use of immunoaffinity resins; some are marketed, which are able to bind high- o medium-abundance plasma/serum components, resulting in the depletion of up to 99% of the total proteins.

~~2.~~2 Without major plasma/serum proteins

~~2.1.~~2.1 How to remove major plasma/serum proteins

~~2.1.1.~~2.1.1 Removing albumin

From the above, removing albumin alone from plasma/serum, and from all biological fluids that derive from plasma/serum (*e.g.* urine and CSF), definitely falls short of significantly enriching the samples in low-abundanc proteins. Still, some specific indications exist for this procedure. The M_r of albumin is close to that of many other plasma/serum components so the already low resolution by mass afforded by 1DE electrophoresis (SDS-PAGE) is furthe limited by the presence of albumin. The p*I* of albumin differs significantly from that of other plasma/serum proteins of like (and unlike) M_r: accordingly, in 2DE with IEF under equilibrium conditions, the spot of albumin is quite distinc from all others. However, using standard protocols with ready-made IPG strips for the 1st d run and in-gel sample reswelling, some of the albumin initially loaded at p*H* < p*I* erratically fails to focus. This results in a horizontal streak i the low p*H* (anodic) region, which overlaps acidic protein spots, blurring their pattern and confusing their quantitation. Cutting albumin concentration also cuts such interference.

A typical approach to remove only/mainly albumin from plasma/serum is through dye-ligand pseudoaffinity chromatography [2,3] on immobilized triazine dyes, most often on Cibacron Blue F3-GA immobilized to give blue resins. With the presence of both fused aromatic rings and sulfate groups, this molecule recapitulates key features of the main physiological albumin ligands (free fatty acids and bilirubin), namely the coexistence of hydrophobic and acidi functions. However, the resemblance is broad, and similarity may be detected also with such an unrelated metabolite as NAD(P). Indeed, high affinity for the blue columns is shared by a whole class of proteins — the NAD/NADI dependent enzymes — that interact with the immobilized dye through their Rossman-folded binding sites. More relevant to plasma/serum proteins, the properties of the immobilized ligand do contribute to both ion exchange an hydrophobic interaction. As a result, the affinity of individual serum proteins for immobilized Cibacron Blue F3-GA changes with ligand density (in different commercial products) as well as with pH and ionic strength of th chromatographic buffer, whether under steady or dynamic conditions (incubation, flow) [4,5]. Moreover, the affinity of albumin itself for the immobilized dyes extensively varies depending on the species [6], being very high for th human protein but from lower to much lower for many of its animal homologs. Accordingly, it is difficult to quantitatively remove albumin (from human and even more from animal specimens) without carrying along other serur proteins; for instance, under most conditions, lipoproteins/apolipoproteins are heavily depleted as well. A high-salt buffer is required to sharpen binding selectivity; as such, without desalting, the flow-through from the adsorptio cartridge/column is not directly suitable for loading in IEF. An assessment of dye-ligand chromatography in connection with plasma proteomics may be found in [7]. In a reverse perspective to depletion, a hydroxyethylthioethyl este adducted tripeptide produced by pronase digestion of albumin was used as quantitative biomarker for the diagnosis of human exposure to sulfur mustard; sample enrichment in human serum albumin by dye-ligand chromatography wa instrumental to improve the performance of the test [8].

Mentioning salts in the previous paragraph reminds a low-tech, old-time approach for separating/removing albumin from other serum proteins, *i.e.* ammonium sulfate precipitation. Albumin is the only major protein soluble a 50% saturation of the chaotrope while its quantitative precipitation calls for 100% saturation. As exemplified by a comparison of immunoglobulin solubility [9], species-specificity sets the conditions for salting-out plasma/serum proteins Indeed, both the depletion of high-abundance components (from rat serum [10]) and the fractional precipitation of (human) plasma proteome with ammonium sulfate (attaining 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, and 55% sa concentrations [11]) have been recently explored as preliminary steps before 2DE analysis.

Another chemical depletion method is based on sequential protein precipitations steps. DTT (50 mM) promotes the precipitation of proteins rich in disulfide bonds, with the formation of a viscous precipitate, mainly containin albumin; the addition with sonication of 2 vol of water and 4.5 vol of acetonitrile to the supernatant results in the precipitation of alpha-2-macroglobulin, transferrin, complement 3alpha (C3), and immunoglobulins. The depleted sampl in the second supernatant may be used in proteomics experiments (Fig. 2) after evaporation of the solvent to dryness [12].

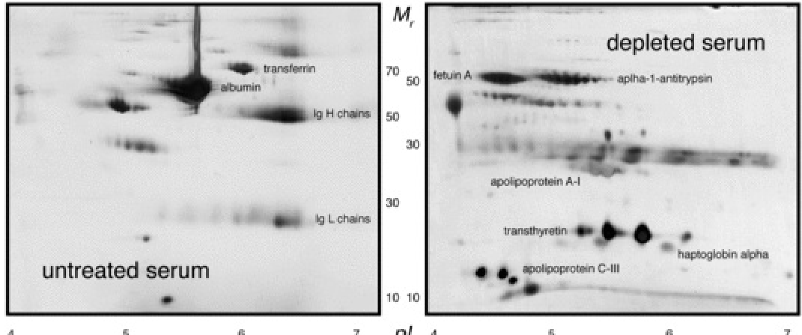

Fig. 2 2DE comparing the different protein profiles of untreated human serum and of serum depleted according to the sequential method in [12]. Protein landmarks are identified in each map.

alt-text: Fig. 2

Still a different type of precipitation procedure is described in [13]: at p*H*(pH (no Italics)) 4.2, salt removal from serum (human, bovine, or porcine specimens alike) reproducibly results in the precipitation of albumin and othe high abundance proteins (immunoglobulins, apolipoprotein A-I, transferrin, alpha-2-HS-glycoprotein, hemopexin, vitamin *D (vitamin D (no Italics))*-binding protein, serpin A3–5, complement factor B).

For some specific applications, excluding albumin and some other major plasma/serum proteins either from analysis or from detection after 2DE may be obtained through simple procedures relying on differences in M_r. Fo instance, in an investigation on (human) amniotic fluid focused on low-M_r proteins and on proteolytic fragments from high-M_r components, we processed proteins (concentrated by freeze-drying) through two steps of size-exclusio chromatography (gel filtration on a Sephadex G100 column) before the electrophoretic analysis [14]. On (rat) serum, we have demonstrated the feasibility of a controlled overloading followed by staining the upper half of the 2ndd sla

with Coomassie, the lower half with silver nitrate, to compensate for the uneven abundance of higher and lower M_r proteins in the sample [15]. Another way to partition proteins on the basis of their size is through (centrifugal ultrafiltration (with MWCO at 30,000), under solvent conditions (25 mM NH_4HCO_3, pH 8.2, 20% (*v*/v) acetonitrile) that hinder protein-protein interactions [16]. In the referred work, the authors did not further fractionate the collecte proteins but treated the whole low-M_r filtrate with trypsin and analyzed the resulting peptides by SELDI.

None of the above procedures actually provides for strict selectivity between albumin and the other plasma/serum components because the fractionation criteria rely on properties shared to different extent by many/all proteins *i.e.* on quantitative rather than on qualitative differences. An all-or-none interaction may be expected instead with such a biospecific device/reagent as an immunoaffinity resin. Some products are commercially available (e.g. an agaros slurry with immobilized *anti*-albumin antibodies, from Sartorius), others may be laboratory-prepared from CNBr- or Protein A-Sepharose gel. Experimental evidence however shows that, together with albumin, a number of proteins ar bound to, and may be released from, an *anti*-albumin resin after plasma/serum immunodepletion (Table 1); their ensemble has been given the name of albuminome [17]. The use of different commercial products results in the co-bindin of a different assortment of non-albumin proteins [18]. Albuminome composition has been assessed also through complementary approaches. In one, human albumin was immobilized to a solid activated immunoaffinity support via coupling reaction. After incubating the resin in a serum dilution and extensive washing, tightly interacting proteins were eluted with a chaotropic solution and identified [19]. In another, the supernatant from 42% ethanol/100 mM NaC precipitation on IgG-depleted serum was processed though size exclusion chromatography and proteins co-eluting with albumin were assessed [17]. All the identified proteins are assumed to interact with albumin *in vitro* as counterpart of their interaction *in vivo*; accordingly, their changes have been investigated as biomarkers of liver disease (hepatitis C virus-related cirrhosis, small unifocal hepatocellular carcinomas and advanced hepatocellula carcinomas) [20].

Table 1 Albuminome components [17].

alt-text: Table 1

Intact proteins	Apolipoprotein A-II Apolipoprotein A-IV Apolipoprotein C-II Apolipoprotein C-III Ceruloplasmin Clusterin Complement component 1 inhibitor Haptoglobin Hemoglobin, alpha chain Hemoglobin, beta chain Hemopexin Leucine-rich alpha-2-glycoprotein Transferrin Transthyretin Vitamin D-binding protein Zinc alpha-2-glycoprotein
Protein fragments	Carboxypeptidase B2 Complement component *C*4 Hornerin Inter-alpha-trypsin inhibitor heavy chain H4 Kininogen Paraoxonase 1 Peptidoglycan recognition protein 2 Plasminogen

2.1.2 Removing major plasma/serum proteins

In agreement with the arguments in Section 1, removing most/all major proteins from plasma/serum is a sounder approach than removing albumin alone to increase the sensitivity of proteomics protocols and to extend detectio and quantitation towards such minor components as tissue leakage proteins. This step is expected to improve the resolution on 2DE gels by enabling visualization of proteins that migrate together with, or close to, the high-abundanc proteins and by increasing the load in the least abundant components.

An attempt to exploit, to this aim, conventional affinity procedures in the analysis of rat plasma can be found in [21]. The authors computed the average hydrophobicity of the major proteins in this specimen ($N = 56$) and sorte them in classes through cluster analysis. The high-abundance proteins turned out to belong to the medium-hydrophobicity class, therefore hydrophobic interaction chromatography (HIC) was rationally designed to deplete them fror plasma. Indeed, when applied before 2DE, HIC permits to detect twice as many spots as immunoaffinity depletion of albumin (above).

A targeted instead of a broad approach implies molecular recognition of each of the components to be removed, which is feasible through immunoadsorption. A number of proposals have been made for laboratory-made as we as commercial products devised to bind a variable number of plasma/serum proteins ($N = 6–20$), both of human and rodent origin. The major advantage of such devices is of course the expected specificity of their interaction; the majo drawback lies in their extremely high cost. Typically, for this application, IgYs [22] directed against plasma/serum proteins are raised in hens during the laying period and purified from eggs (structure in Fig. 3). Production of IgYs i minimally invasive on the immunized poultry, which complies with the current guidelines on animal welfare. Depending on the antigen, Ig titer development in chicken may compare more or less favorably with rabbits or mice. Chicke Igs are clearly superior to rabbit Igs in terms of epitope recognition when the antigen is a protein of mammalian origin, whether human or animal. Such an advantage in terms of higher specificity/lower cross-reactivity and highe sensitivity is obviously connected with the higher phylogenetic distance between self and immunizing non-self in chicken than in mammals. Among the commercial products, it is worth mentioning those from Agilen (https://www.agilent.com/), from R&D Systems (https://www.rndsystems.com/) and from Sigma (http://www.sigmaaldrich.com/). They include two classes of depletion columns: one targeting 12 or 14 of the high-abundance proteins another the middle-abundance proteins; the polyclonal polyspecific antibodies for the latter are obtained through immunization with the flow-through from the former. The sequential use of both columns is intended to leave in solutio only low-abundance proteins.

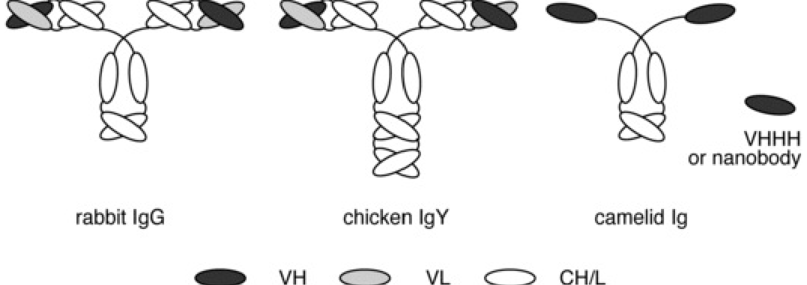

Fig. 3 Overview on the structure of immunoglobulins of different animal origin.

alt-text: Fig. 3

A related approach to ‘ultradepletion’ of human plasma was put to the test by fractionating plasma with dual ion exchange columns, producing polyclonal IgY against each fraction and using the purified antibodies in a immunodepletion column. A total of 165 non-redundant proteins were identified after depletion; of these, 38 had not previously been identified in non-depleted plasma [23].

Camelids produce functional antibodies devoid of light chains; antigens are bound by the *N*-terminal variable domain of the heavy chain (see Fig. 3). The corresponding single-domain antibody fragments (VHHs or nanobodies, i Fig. 3) are fully capable of antigen binding, with affinities comparable to conventional antibodies. VHHs are efficiently produced in *Saccharomyces cerevisiae*. With their high stability and solubility, these affinity reagents have severa advantages for biotechnological applications [24]. One commercial device using them to capture 14 high-abundance proteins is produced by BAC/Bionity (http://www.bionity.com/en/companies/15295/bac-b-v.html). An example of it application may be found in [25] (Fig. 4): removal of high-abundance proteins is efficient and enrichment of low-abundance proteins appears reproducible from run to run.

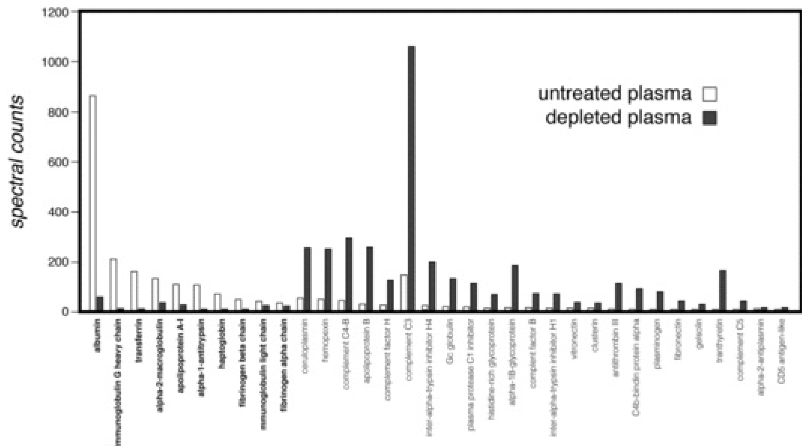

Fig. 4 Relative abundance of proteins identified in untreated plasma and in the flow-through fraction of an immunoadsorption column targeting 14 major plasma components (bold). (Redrawn from [25]).

alt-text: Fig. 4

Due to the complexity of the immunological approach, several reports have been devoted to the assessment of a number of issues. The efficiency of immunosubtraction was first evaluated for devices meant to remove 6 higl abundance proteins (albumin, IgG, IgA, transferrin, alpha-1-antitrypsin, and haptoglobin), comparing the 2DE pattern of serum before and after removal of these proteins. One paper rated the yield through the number of detectabl spots: with sample treatment, the count grew from approximately 850 to over 1500, with a 76% increase [26]. In another paper a criterion for quantitative comparison was identified in the number of matched spots (applying an imag analysis software to the 2DE patterns of several replicates); the matches totaled 197 before and 317 after depletion, with an increase of 61% [27] (Fig. 5).

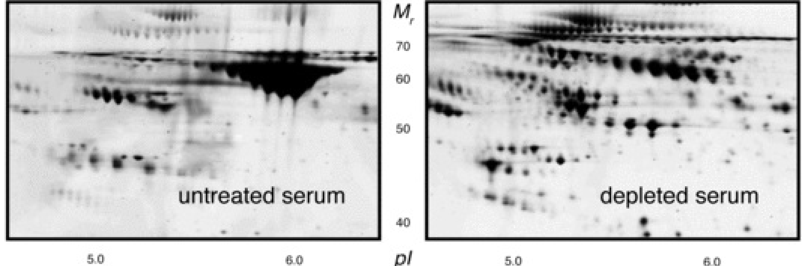

Fig. 5 2DE comparing the different protein profiles of untreated human serum and of serum immunodepleted in six high-abundance proteins (albumin, IgG, IgA, alpha-1-antitrypsin, transferrin, and haptoglobin). (From [27]).

alt-text: Fig. 5

The next issue to be addressed was the cost-effectiveness of devices meant to deplete from plasma/serum an increasing number of abundant proteins. With a title asking: The more, the better? [28], a paper tries to provide a answer to this question by comparing the results obtained after depleting 1 (albumin), 6 (albumin, IgG, IgA, transferrin, alpha-1-antitrypsin, and haptoglobin, see above), 12 (the previous *plus* apo A-I and -II, α_1-acid glycoprotein, alpha-2 macroglobulin, fibrinogen, IgM) or 20 proteins (the previous *plus* IgD, ceruloplasmin, apo B, complement C1q, C3, *C*4, plasminogen, and transthyretin). After analysis by 1DE and 2DE and SELDI-TOF, the outcome is not univocal. Th gain is substantial removing 12 proteins instead of 6, limited removing 20 proteins instead of 12. As already mentioned for albumin and its albuminome, while adsorbed under non-denaturing conditions the immunocaptured protein remain associated with a whole range of peptides and proteins, which are removed alongside (off-target effects) (Fig. 6). A largely similar experimental design was implemented in another paper [29]. In this case, the systemati comparison involved the immunodepletion of 6, 14 (the two extra proteins bound *vs* the 12 above are C3 and transthyretin), or 20 proteins; the analytical procedures included both top-down (2D-DIGE) and bottom-up (LC-MS/MS approaches. Using the combination 1-DE–LC-MS/MS, the number of Z2 unique peptides rose from 159 in unfractionated serum to 301 following depletion of 20 proteins. When comparing the results after immunoadsorption of eithe

14 or 20 proteins, additional spots were seen in 2DE but most of them were isoforms of already detected proteins; moreover, a greater run-to-run variability was observed in LC-MS/MS after more extensive depletion (media %CV = 30.9 vs 18.2%). The removal of either 6 or 14 abundant components was tested also on the proteins of such peculiar specimens as the mechanically induced skin suction blisters, whose fluid may serve as model sample fo dermatological diseases [30,31] (Fig. 7). Analysis of the tryptic digests by 2D-LC (reversed-phase chromatography at high and low pH after iTRAQ derivatization) associated the depletion of the top 14 components with a lower efficienc on the target proteins, yet a higher enrichment factor, a higher number of identified protein groups, an improved dynamic range and a higher loading capacity [31]. Finally, from a further paper [32], we like to quote quantitative data o the binding properties of the Sigma resins meant to deplete 14 high-abundance and a number of medium-abundance plasma/serum components: their use removes at least 155 proteins, 38% of the plasma proteome in protein numbe and 94% of plasma protein in mass. The authors question on the weakness of such extreme immunodepletion (split line at immuno-depletion (see pdf)) procedure in front of even slight variations in inter-assay binding efficiency: they argu that the proteins associated with the highest percentage immunodepletion are also at the highest risk of being identified as false-positives in differential proteomic studies.

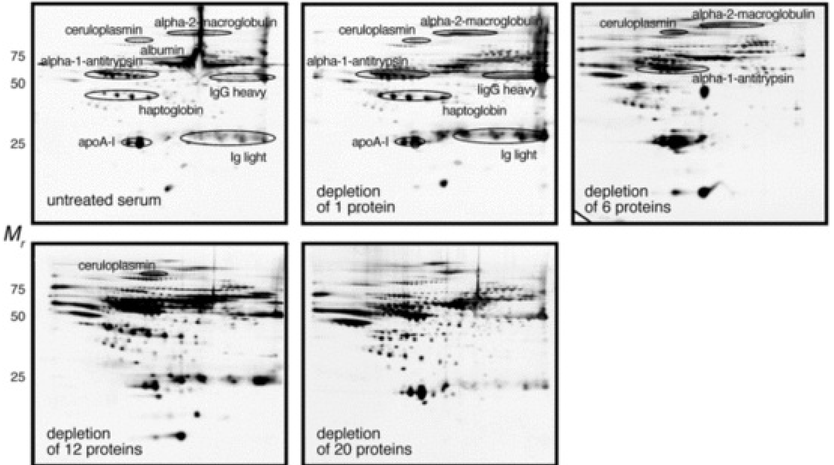

Fig. 6 2DE of untreated serum *vs* the unbound fractions collected after depletion of 1, 6, 12 and 20 major serum proteins using commercial columns (Table 1). A fixed amount of protein (50 µg) was analyzed in all maps. (From [28]).

alt-text: Fig. 6

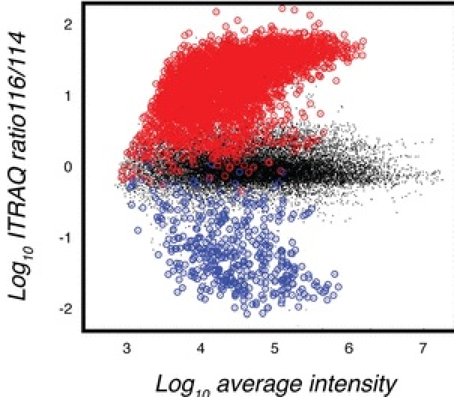

Fig. 7 Scatter plot of Log_{10} iTRAQ ratios *vs* Log_{10} average intensities for all peptide-spectrum matches. Blue circles correspond to the 6 most abundant proteins (albumin, IgG, IgA, transferring, haptoglobin, alpha-1-antitrypsin), and red circles include the additional 7–14 most

abundant proteins (IgM, alpha-2-macroglobulin, fibrinogen, complement C3, alpha-1-acid glycoprotein, apolipoprotein A-I/II (HDL), and apolipoprotein B (LDL)). (From [31]).

alt-text: Fig. 7

As a last line, similar to albuminome, the term depletome has been introduced to refer to the off-target immunosubtracted proteins (see above).

2.2 How to enrich minor plasma/serum proteins

An opposite perspective to depletion of high-abundance proteins from plasma/serum is the enrichment of low-abundance ones through an increase of their relative concentration; such a compression of the dynamic range i expected to provide a deeper proteome profiling. This technology was described by the inventors as “protein equalization” and its effect as approaching a “democratic proteome” [33]. The enrichment procedure is based on a specifi and saturable interaction of proteins to a high diversity of binding sites exposed on chromatographic beads. The combinatorial ligand library is made up of dozens of millions of hexapeptides capable of interacting with most, if not al proteins in any given proteome [34,35] (Supplementary Figs. 1 and 2). BioRad (http://www.bio-rad.com/) markets the corresponding commercial kit, ProteoMiner, together with 4 elution reagents meant to sequentially recover protein on the basis of different properties, in a format compatible with analysis by SELDI. Differential elution under native conditions may be achieved by changing the pH of the buffer [36] or by using a mixture of charged aminoacid (150 mM Lys, Arg, Asp and Glu) [37]. Elution of all bound proteins under denaturing conditions is made possible by boiling the beads in SDS [38,39]; precipitation with acetonitrile then provides for the highest protein recovery yield an the best 2DE spot pattern [40].

The conceptual background to the peptide ligand library capture is a saturation effect: all the proteins present in the starting sample in concentrations high enough to overload their interaction sites on the beads should b bound in equal amounts, the excess being discarded with the flow-through. After stripping the bound proteins from the resin, the concentration of the higher-abundance proteins should be equalized in the eluted fraction. Conversely, th proteins present in the starting sample in concentrations too low to overload their interaction sites on the beads should be quantitatively bound. Accordingly, the differences among samples should be intentionally abolished (or at leas reduced) for higher-abundance and preserved for lower-abundance proteins. A test of the quantitative performance of the capture beads shows an average variability around 10% in the amount of the isolated proteins, independent c their initial abundance. No normalization effect is in fact observed but, depending on the protein-to-bead ratio, specific sets of proteins are either enriched or depleted *vs* the starting whole proteome [43] (Supplementary Fig. 3). A reports that evaluate the performance of the protein enrichment strategy [44] or compare it to depletion protocols [45] acknowledge the expected compression of the dynamic range of serum protein concentrations.

To further reduce sample complexity, equalization has been combined with additional chromatographic steps on IMAC and RP columns (tandem monolithic columns with surface-bound iminodiacetic acid ligands saturated wit Zn^{2+}, Ni^{2+} and Cu^{2+} connected to a reversed-phase column packed with polystyrene divinyl benzene beads) [46]. An alternative to the hexapeptide ligands has been proposed in the form of single chain variable fragment displaying M1 phage library [47]. Both immobilization on beads and elution of bound proteins require specific precautions not to interfere with the structure of the phage; the number of proteins identified from human serum with such an enrichmen procedure is twice as high as from the untreated sample.

The enrichment procedure may be applied to any kind of sample of vegetal or animal origin. Focusing on human specimens, plasma/serum has been used most often both when setting up the procedures and when analyzin actual samples for marker discovery. However, also other body fluids have been processed this way, including cerebrospinal fluid [48], milk/milk whey [49,50], saliva [51], seminal plasma [52], artificial peritoneal dialysis effluents [53 synovial fluid [54].

The pathological states mainly investigated were neoplasias (pancreas [55], lung in never-smokers [56] and of the non-small cell type [57,58]) and infections (hepatitis B-associated liver cirrhosis [59], HIV-1/HCV mono- and cc infection [60], invasive aspergillosis — in a mouse model [61]). Two further topics of peculiar interest addressed with this technique were the changes occurring in serum with gestational ages (for infants with intrauterine growt restriction [62]) and those occurring under extreme physical stress [63].

An alternative enrichment approach based on physicochemical properties of the proteins rather than on their binding affinities is being proposed in [64]. This method, which targets hydrophobic proteins, consists of a phas separation with Triton X-114. The detergent-rich phase is collected after raising the temperature to above the cloud point, then diluted at a temperature below the cloud point and concentrated by a TCA-acetone precipitation step. In comparison with analysis on whole serum and on serum processed through albumin and IgG immunodepletion and protein enrichment on hexapeptide resin, the alternative approach allowed to recognize statistical significance c differences between control and test animals (pigs exposed to feed supplementation with sub therapeutic levels of oxytetracycline) for three hydrophobic proteins, namely apolipoprotein F, clusterin and paraoxonase.

3 With major plasma/serum proteins: How to deal with whole plasma/serum

3.1 Strategies in proteomics

All we have presented so far shares a perspective: differences in minor components are sought in plasma/serum after simplifying the composition of the sample. Removing major components is the primary strategy; furthe

simplification may be obtained with any fractionation procedure while the final resolution into sizeable analytical units is obtained with 1D- or, more usually, 2D-procedures, either electrophoretic or chromatographic. Investigation along this line are not guided by previous knowledge: statistical significance of differences in abundance is the only selection criterion. In this sense, these investigations fully comply with the definition of proteomics as an unbiase qualitative and quantitative evaluation of all protein components in a given sample ~~–~~— sample in this case being human plasma/serum.

To overcome the difficulties along this track, more and more often the proteomic investigation on human pathologies is being moved from plasma/serum to tissues. Proteins whose concentration is significantly altered under th test *vs* the reference conditions are then measured in plasma/serum of the same subjects to verify whether the components of interest are present at a level adequate for reliable quantitation. When this is the case, the circulatin concentrations are evaluated and statistical significance of differences is finally assessed.

However, human specimens other than few body fluids require invasive procedures for their procurement, which are often neither practicable nor ethically acceptable. Moreover, due to genetic and environmental influences sampling conditions from human subjects are seldom comparable, which increases biological diversity in any test population. For these reasons, in many cases, the simple scheme above is better extended from a two- to a multiste procedure. Proteomic analysis at the tissue level is carried out on animal models of disease *vs* relevant controls. Differential proteins are then sought in human tissue sections, to verify whether in the human species the same difference in abundance between test and control samples are present as in the animal model. Only after this check the quantitation on human plasmas/sera is eventually carried out for the putative biomarkers.

While the discovery phase of this alternative strategy implies tools and procedures typical of proteomics, the validation phase usually completely relies on immunological reagents. The quality of the latter is crucial in the whol process but producing/screening high-specificity high-affinity antibodies, whether polyclonal or monoclonal, is not an easy task.

Overviews on integrated strategies for biomarker discovery and validation have been published (e.g. [65]). All of these aspects are dealt with, through relevant examples, in the following sections.

~~3.1.1.~~3.1.1 *Reference immunological reagents*

The limited number of available specific antibodies in spite of a multitude of efforts in the academic and industrial sectors led the Human Proteome Organization (HUPO) to endorse antibody-based proteomics programs involvin the systematic generation of antibodies for use in functional exploration of the human proteome with a high-throughput approach [66,67].

This endeavor has been given the name of Human Protein Atlas (http://www.proteinatlas.org/about); it was started at the Royal Institute of Technology, Sweden, in 2003 and is funded by the non-profit organization Knut and Alic Wallenberg Foundation. Updates on the program achievements are since published on a regular basis (e.g. [68,69]).

The in-house antibody production process begins with a bioinformatics analysis of the protein-coding part of the genome. For every protein, the amino acid sequence is compared to all other putative protein-coding genes t identify a stretch of 50~~-~~–150 amino acids that has as low homology as possible with respect to all other proteins. These sequences are then cloned from cDNA libraries using specifically designed primers and transformed into ~~coli~~*Escherichia coli* bacteria that produce the corresponding peptide chain (protein epitope signature tag or PrEST). The PrEST is used for various applications including immunization to produce antibodies, and for affinity purifying th polyclonal antisera to make them strictly monospecific. Numerous quality assurance and validation steps are performed to eventually certify the antibodies. The same validation protocol is applied also to commercial products and t antibodies prepared by collaborating institutions.

By copying in the above paragraphs most of the basic information about the pipeline of antibody production summarized in the Atlas web site, we want to emphasize that, in order for the quantitative results from in solution o from on membrane immunodetection procedures to be meaningful, the quality of the immunological reagents *must* be certified through a complex and stringent procedure (typically by external bodies) and the calibration curves for th assay be carefully built (in house). Sometimes the differential item corresponds to just one of a number of species deriving from a single polypeptide through differential PTM processing (see Section 4.1). In these cases the copy numbe per cell of the protein (all species) is affected much less than that of the individual species; a measure of the protein as a whole, by ELISA or by immunoblotting after 1DE, would likely miss statistically significant differences (a exception, 1DE after extensive proteolysis). Such cases can be effectively addressed only through 2DE with immunoblotting and comprehensive image analysis.

~~3.1.2.~~3.1.2 *Reference data on tissue proteomics*

The baseline level of expression for each individual protein in each tissue is relevant information when changes under pathological conditions are evaluated. Therefore we go on reporting basic information about the Protein Atla project.

Protein expression profiles are produced for each of the certified antibodies by staining different standardized sets of biological samples from 44 different normal human tissues, 20 different cancer types, 46 different human ce lines and 6 hematopoietic cell types from patients. As of spring 2016, proteome analysis has been carried out based on 25,039 antibodies, targeting 17,005 unique proteins [69]; results are available on line, in a searchable web sit (http://www.proteinatlas.org/about) that also contains transcriptomics data on the mRNAs coding for the same proteins. Information is divided into a Tissue Atlas, a Subcellular Atlas, a Cell Line Atlas and a Cancer Atlas.

In detail, the tissue microarrays for high-throughput immunohistochemistry consist of multiple 1-mm diameter cores from formalin-fixed paraffin-embedded tissue specimens, arranged in a single paraffin block. Each block i cut into 200–250 sections to be used for separate immunohistochemical staining experiments; processing several arrays under similar conditions sizably reduces intra-experimental variation. The stained slides are then scanned an pathologists annotate each core image by scoring the intensity of immunoreactivity with its cellular localization and by counting the fraction of immunostained cells.

~~3.1.3.~~3.1.3 *Multistep experimental plans*

From the first proposals to the most recent experiences, increasing levels of complexity may be found in different experimental plans, shifting between tissues and body fluids and between human and model animal specimens.

A breakthrough in this area was the use of xenografts, *i.e.* immune-incompetent nude (mutation in *Foxn1* gene) or SCID (mutation in *Prkdc* gene) or RAG-2/γ(c)KO (H-2d RAG2 −/−IL-2Rγ −/−, or RAG2 −/−γc (gamma common)−/− mice transplanted with human tumor cells. Proteins secreted (or shed) by the tumor can be identified in mouse circulation and their human derivation confirmed. The xenograft setup provides a much more favorable ratio of the relevan human tumor-derived biomarkers *vs* the irrelevant mouse plasma/serum protein background. Examples of this approach include the following reports, in which either established cell lines or pathological material derived from individua cancer patients are grafted.

As for the established lines, PC339 cells provide a model of androgen-independent human prostate cancer; tumor-derived human nm23/nucleoside-diphosphate kinase and six human enzymes involved in glycolysis were identifie in mice bearing the xenograft [70]. A hepatocellular carcinoma model may be obtained by injection of HCCLM3 cells; the levels of circulating cytokeratin 19 and of its fragment CYFRA 21-1 were found associated with metastasi potential [71]. Three cell lines (liposarcoma [SW872], osteosarcoma [KHOS-24OS], and mammary adeno-carcinoma [MDA-MB-436]) exist in two, non-angiogenic (microscopic, dormant tumors) or angiogenic (rapidly growing tumors), phenotypes; analysis of the corresponding xenografts suggested tha platelet-associated platelet factor-4, but not its plasma counterpart, may represent a potential biomarker of early tumor presence [72]. Grafted MKN45 gastric cancer cells were found to release in mouse plasma human apoA-I; its leve was lower in mice with large than in those with small tumors [73].

As an example of biopsy-derived cell lines, specimens from both human oral squamous cell carcinomas and adjacent control tissue were transplanted orthotopically into mouse tongues; several proteins were found to differ i concentration between control and cancer-bearing mice; among these, EGFR levels inversely correlated with the invasive phenotype [74,75].

The first type of advance from the above scheme was the validation in human sera of the findings in the xenograft mouse models. For instance, transplant of NPC-TW02 cells, derived from a keratinizing nasopharyngea carcinoma, resulted in elevated circulating levels of peroxiredoxin 2 and carbonic anhydrase 2; similar results came from the analysis of plasma from nasopharyngeal carcinoma patients [76]. Ninety-seven proteins were identified in th culture supernatant of the ovarian cancer cell line SKOV-3. After the cells were xenografted into the peritoneal cavities of nude mice, three of these proteins were detected in animal sera; one of them, 14-3-3 zeta, was identified as candidate biomarker through an ELISA-based screening of clinical blood samples. The average serum levels of 14-3-3 zeta were higher in patients with epithelial ovarian cancer than with benign gynecological diseases and correlate with clinical parameters of disease severity [77]. Using a different ovarian cancer cell line, TOV-112D, ~~more than 200~~> 200 human proteins were identified in the xenograft serum; three of them (chloride intracellular channel 1, cathepsi D, and peroxiredoxin 6) were then found elevated in sera from ovarian carcinoma patients [78].

A similar shift in plans applies to a different type of animal models, the genetically engineered mice.

Both sera and tissue samples were compared from Pdx1-Cre *Kras*^{G12D} *Ink4a/Arf*$^{lox/lox}$ mice as models of pancreatic cancer and from *Kras*^{G12D} *Ink4a/Arf*$^{lox/lox}$ and Pdx1-Cre *Ink4a/Arf*$^{lox/lox}$ control animals. A panel of five protein selected on the basis of their increased level at an early stage of tumor development in the mouse (neutrophil gelatinase-associated lipocalin, lithostathine-1-alpha, regenerating islet-derived protein 3-alpha, metalloproteinase inhibitor 1 and insulin-like growth factor-binding protein 4) was tested in a blinded study on 26 subjects with increased cancer risk (from the large cohort of > 18,000 individuals enrolled in the Carotene and Retinol Efficacy Trial, CARET). Th panel discriminated pancreatic cancer cases from matched controls in blood specimens obtained between 7 and 13 months prior to the development of symptoms and clinical diagnosis of pancreatic cancer [79]. Plasma data from comparison between AdenoCre-infected K-ras/Pten mice as an ovarian cancer model and Adeno-empty injected controls were evaluated against evidence from three human ovarian cancer cell lines (OVCAR3, CAOV3, and ES2) and fro cells in the ascites fluid of an ovarian cancer patient. A set of proteins (insulin-like growth factor-binding protein 2, metalloproteinase inhibitor 1, retinoic acid receptor responder protein 2, monocyte differentiation antigen CD14, an granulin) were measured at higher levels in pathological than in control tissues and body fluids, both human and murine. The difference from controls was significant from the early stages of the disease [80].

An example not related with cancer involves a transgenic mouse model with cardiac-specific overexpression of activated calcineurin (CnA), which results in severe cardiac hypertrophy. Four proteins (myosin heavy chain 7 insulin-like growth factor-binding protein 7, annexin A2, and desmin) were found significantly different in the CnA hearts compared to controls and overexpressed in proteomic and transcriptomic dataset of heart failure. Immunologica quantitation in mouse and human sera demonstrated that all four proteins increased between twofold and 150-fold in heart failure [81].

3.2 Procedures in proteomics

Both optimal and maximum load in a separation procedure is a function of the size (length, surface, or volume) of the separation medium during its limiting step. In 2DE, the volume of the 1[st]d IPG strip controls the total amoun of proteins to be processed *per* test. Using standard ready-made commercially available strips, the amount of proteins/the volume of whole (and as well as of depleted) plasma/serum to be run is also standard.

Using standard ready-made commercially available IPG strips is the most common procedure in IEF for practical reasons that include both the ease of the current simplified protocols and the reproducibility of the substrate fo the separation procedure, which entails the reproducibility of the results. These obvious advantages should however be weighed against a few disadvantages, lack of flexibility being the most evident and the most stringent.

From the early history of IPGs [82] we are accustomed to polymerize our own gels, in all kind of pH gradient, and to cut strips from them, of all sizes and shapes [15]. This makes it very easy to load very large volumes of whol serum; the only precaution, already recalled in 2.1.1, is to apply the sample near the cathode, since running through regions with pH lower than p*I* most often results in substantial albumin streaking, as first demonstrated by us back i 1985 [83]. Conversely, exposure to acidic pH of much of the albumin content of a sample cannot be avoided when proteins are loaded through the in-gel rehydration procedure that is customary with the commercial IPG strips.

Even for animals the size of a mouse —few tens of grams in weight —tens of microliters of whole plasma/serum are most often available for testing. This allows for each sample to be analyzed under more than on experimental condition. Using 2DE, obvious variations to the basic procedure aim at focusing on one or more areas in the pI/M_r plane via zooming-in *i.e.* by narrowing the range of protein charge and mass to be covered at hig resolution. However, a less frequently taken but highly effective alternative is to run each sample both under reducing and under non-reducing conditions. As we have demonstrated for a number of species, humans and animals as wel this change in experimental protocol significantly modifies the migration position not only of disulfide-associated multi-chain assemblies, which split into their constituent subunits, but also of disulfide-bridged single-chain proteins whose unfolding in the presence of SDS is restricted by the presence of covalent bonds [84].

The following observations, taken from [84], summarize our findings about the major serum proteins in four species —*Homo sapiens* (Fig. 8), *Rattus norvegicus*, *Mus musculus*, *Bos taurus* —and stress advantages an disadvantages of alternative procedures. Under non-reducing conditions albumin of all species migrates to a much lower M_r than under reducing conditions: 55 *vs* 68 kDa, consistent with the presence of 17 S-S bonds. Either because c better recovery or higher affinity for CBB stain, the spot for non-reduced albumin is larger and darker. Polymeric forms on top of a diffuse vertical streaking are also observed. Unreduced conditions allow for the best resolution betwee albumin and hemopexin in human, mouse and rat serum samples. Albumin fragments are present in different amount in various species. Where they are prominent (rat and mouse serum) detection is possible only after reductio —which implies the presence in rat and mouse circulation of a substantial amount of cleaved but undissociated albumin.

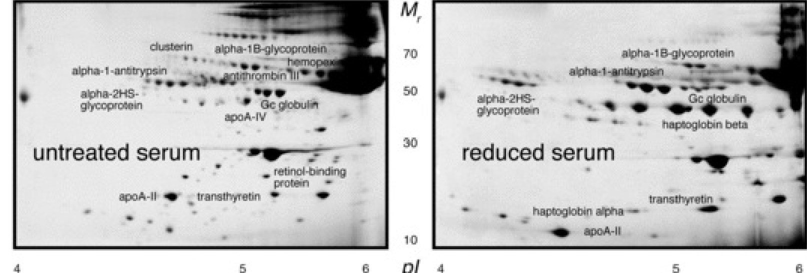

Fig. 8 2DE pattern of serum from a control subject run under non reducing (left) *vs* reducing conditions (right). Our unpublished results.

alt-text: Fig. 8

Transferrin also contains a large number of S-S bridges (19) and its M_r shifts from 71 to 77 kDa between non-reducing and reducing conditions. Bovine serum contains two different molecular forms of transferrin, which diffe by the presence or absence of a *C*-terminal peptide of about 6 kDa [85]. The resolution between the two is minimal under non-reducing conditions but increases on reduction. The charge microheterogeneity of native transferrin i connected to both covalent post-transcriptional modifications (glycosylation, implying up to 8 sialic acid residues) and non-covalent interactions (binding of up to 2 Fe^{++} ions). The susceptibility to urea denaturation varies depending o iron saturation and redox status. The diferric protein is unaffected by urea, and in the monoferric transferrin only the iron-free domain is assumed to unfold [86]; in ovotransferrin, the molecule containing 4 S-S bridges is still able t bind iron, and the corresponding complex doesn't unfold in 8 M urea, whereas the 3 S-S and 2 S-S proteins exist only as apoforms [87]. Accordingly, more proteins species are resolved before than after S-S reduction.

The amount of immunoglobulins varies among species, being much higher in humans and *Bovidae* than in *Rodentia*. As expected, without sample reduction Igs form horizontal streaks at high M_r whereas after reduction heav and light chains are resolved at lower M_r.

Baseline levels of haptoglobin also vary among species, being higher in humans and mice. In these serum samples, alpha and beta chains are resolved after sample reduction but no intact haptoglobin is observed in the 2DE map probably as a result of aggregation. In contrast, in rat serum a distinct if faint series of high M_r haptoglobin spots is seen under non-reducing conditions. Possibly due to their low abundance once separated, both alpha and beta chain are hardly detectable in rat serum after sample reduction. In rats and bovines, noticeable levels can only be detected in case of inflammation [88,89]. In addition, haptoglobin appears in different phenotypes and polymorphism depending on the species [90].

Apolipoprotein A-II is a homodimer in humans and a monomeric protein in the other species. Accordingly, the M_r of apoA-II in human serum is twice as high under non-reducing than under reducing conditions.

The most crowded area in serum 2DE patterns is the one where several 'alpha-globulins' migrate — p*I* ca. 4.5–6 and M_r ca. 50–70 kDa. In human and rat samples the intrinsic differences in p*I* and M_r between adjacent spo rows under reduced conditions are sufficient for discrimination of all components with minimal overlaps. Full-range IPG and SDS-PAGE are adequate for quantitation of all serum proteins and zooming is an aid, not a requirement, fo resolution in the 'alpha-globulin' area of these species. With mouse serum the reduced proteins migrate in closer proximity, and zooming becomes more valuable. In reduced bovine serum a number of proteins concentrate in a extremely narrow M_r range while forming a continuum of charge distribution with few sharply defined spots: even with specific PAA gradients for finer size resolution the discrimination among proteins is thus very poor.

For all serum samples the situation in terms of overall resolution over the specified 'alpha-globulin' area is at least as good under non-reducing as under reducing conditions — and in some cases it is more favorable to run th proteins unreduced or partially reduced. This is especially the case with bovine serum: for its analysis it seems appropriate to use a two-step strategy, namely a run under reducing conditions on a wide range 2DE gel followed by a ru under non-reducing conditions on a narrow-range 2DE gel (e.g. pH 4–6 IPG, SDS-PAGE on 6–12% PAA). The second step is adequate for the evaluation of the 'alpha-globulins', the first step for the evaluation of the other proteins (lov M_r, or alkaline p*I*).

4 Focus on major plasma/serum proteins

Much of what we have described so far has as an explicit or implicit rationale the quest for optimal procedures to detect and quantify minor serum components, most of which derived from peripheral tissues a a result of pathological events. In this perspective, major serum proteins are regarded as a worthless burden. In the following we propose a different, or at least complementary, view.

The point we want to make is that information provided by abundant proteins should not be overlooked, mainly when dealing with a poorly characterized naturally occurring condition or with a newly devise experimental model. Ideally, in our opinion, the survey on whole plasma/serum should always be the first step of a multistage investigation, trying to address, one after another, qualitative and quantitative features c different sets of test proteins at increasing levels of resolution.

4.1 Protein species

Each protein may exist in various molecular forms as the compound result of several types of PTM [91]. Changes in the relative abundance of these protein species reflect the varying activity of the enzymes involved i alternative PTM processing (two recent review articles on this topic as applied to *e.g.* neurodegeneration and autoimmune disease are: [92,93]). The activities of the modifying enzymes are in turn affected by a number of physiologica and pathological factors: data from the assessment of individual protein species may thus provide clues to the attendant conditions.

4.1.1 Proteolysis

Albumin is present in plasma/serum in forms that differ by size — proteolytic fragments on the one side, polymers on the other. The former are detectable only under reducing conditions, the latter only under non-reducin conditions (Section 3.2). In normal human plasma, albumin fragments have M_r around 45, 28 and 19 kDa and make up < 2% of total albumin. The oxidant environment of the extracellular milieu prevents fragment dissociatio to occur *in vivo*: indeed, the ratio fragments *vs* intact albumin is not higher in urine than in plasma/serum samples from the same subject.

Chymase is a chymotrypsin-type serine protease stored in the intracellular granules of mast cells until liberated by degranulating stimuli. Proteolysis of its substrates is part of the inflammatory response: processing c angiotensin I to angiotensin II supports blood pressure; degradation of inflammatory cytokines and other bioactive peptides limits the spread of the reaction; activation of procollagenase to collagenase contributes to extracellular matri remodeling and to resolution of tissue injury [94,95]. Chymase recognizes in albumin the target sequence RETY to cleave it at Y84 and produce a larger, ca. 59 kDa, and a smaller, ca. 10 kDa, fragment [96]. However, as the tw fragments do not dissociate (because of the C75-

S-

S-

C91 covalent bond) nicking by chymase has no influence on the properties of albumin, including cation binding (Cu^{2+} or Zn^{2+}) at a site near the cleavage point [96]. Accordingly, the *in vitro* demonstration of anticoagulan properties for *S*-carboxymethylated CNBr-fragments of albumin is unlikely to have any *in vivo* counterpart, and not only because of the unphysiological type of cleavage [97].

A peculiar type of nicking is involved in the antiprotease mechanism of serpins, as exemplified by alpha-1-antitrypsin. A protease, most often elastase from granulocytes, docks with the exposed reactive loop of alpha-1 antitrypsin, because its sequence and conformation resemble a model substrate. After cleavage at the 'bait' peptide bond (M358-S359), the reactive loop rapidly inserts within an underlying beta-pleated sheet motif as an extra, centra beta-strand. In the process, the catalytic machinery of elastase is distorted and the enzyme cannot complete the catalytic cycle and regenerate to the free, active form from the reaction intermediate (in which elastase acylates S359) The thermodynamic changes associated with the conformational rearrangement of alpha-1-antitrypsin from a metastable (S, for stressed) to a stable (R, for relaxed) conformation are highly favorable, and allow the couplec thermodynamically unfavorable, distortion and trapping of elastase to take place [98,99]. The occurrence of circulating elastase–alpha-1-antitrypsin complexes has proven to be a most sensitive indicator of granulocyte activation i sepsis and trauma [100,101]. As such it has for instance been evaluated as a marker of unwanted side effects of exogenous surfactant replacement for immature preterm neonates with respiratory distress syndrome [102]. Baseline level of the complexes are around 0.1 μg/mL to increase a few folds during granulocyte activation [103]; their quantitation is made by ELISA.

Thyroxine-binding globulin (TBG) is a non-inhibitory serpin. Cleavage by elastase results in the loss of a 4- to 5-kDa *C*-terminal fragment and a major conformational change that weakens the affinity for T_4 [104]. The proteolyti fragments are observed in sera of septic patients [104] but also during the rapid decrease of TBG following cardiopulmonary bypass [105]. The fall in concentration to a mean level of 60% *vs* the preoperative control at 12 h seem thus connected with the local inflammatory response to the surgical procedure and results in the accumulation of free T_4 at the inflamed sites [105].

We have described ourselves how apolipoprotein A-I in HDL is a target of the matrix metalloproteinases released from activated macrophages. The fragments, of 26, 22, 14 and 9 kDa in size, result from both *N*- and *C*-termina cleavage and dissociate from bound lipids. HDL_{3C} (small pre-beta HDL) are more extensively degraded than the other HDL subclasses, with adverse effects on reverse cholesterol transport in which the smallest lipoprotein particles ar specifically involved [106]. We could detect i*n vivo* proteolytic fragments of apolipoprotein A-I in some patients with acute myocardial infarction, likely as a result of the same activation events that in these subjects lead to plaqu fissuration [107]. Moreover, we observed such fragments also after thrombolysis with (recombinant) tissue-type plasminogen activator. This therapy was an early breakthrough for the treatment of coronary occlusion. Once percutaneou coronary intervention became a feasible alternative, thrombolysis was sometimes associated to supposedly facilitate it. Unexpectedly, however, the benefit of facilitated angioplasty turned out to be reduced in comparison with the nor facilitated intervention. Degradation of circulating HDL may provide a potential mechanism for this outcome.

Bikunin is a 25 kDa proteinase inhibitor present in human plasma covalently linked, through a glycosaminoglycan chain, to one or two homologous heavy chains, forming high molecular weight proteinase inhibitors called pre alpha- and inter-alpha-inhibitor. During inflammation, bikunin synthesis is down-regulated, still its excretion in urine increases. This apparent inconsistency is connected with neutrophil activation and the cleavage of the inhibitor fror the *C*-terminus of the heavy chains by the released proteases [108].

C-reactive protein or CRP, the main positive acute phase reactant protein in humans, displays several functions associated with host defense: it promotes agglutination, bacterial capsular swelling, phagocytosis and complemen fixation through its calcium-dependent binding to phosphorylcholine; it can scavenge nuclear material released from damaged circulating cells interacting with DNA and histones. CRP is part of a complex homeostatic mechanism tha governs the acute phase reaction, regulating the immune response and the activity of matrix-degrading enzymes. It is noteworthy that different properties are displayed by the whole pentameric assembly as well as by some proteolyti fragments. For instance, while native CRP does not induce phagocytic leukocytes to chemotax or to produce superoxide, treatment of purified CRP with human neutrophil-derived acid proteases produces substances with potent effect on leukocyte function. As a likely explanation, the sequence of CRP contains peptides closely resembling the immunomodulator tuftsin [109]. In contrast to the inability of intact CRP to inhibit either human leukocyte elastase or huma leukocyte cathepsin G, both associated with chronic inflammatory tissue damage, the peptide 62–76 from the inner disulfide loop of human CRP inhibits these enzymes at concentrations far lower than the acute-phase concentration c the protein [110]. CRP peptide 201–206 induces *L*-selectin shedding from human neutrophils and inhibits *L*-selectin-mediated neutrophil adhesion to TNFalpha-activated human coronary artery endothelial cells under non-stati conditions. It also attenuates shear-induced up-regulation of platelet *P*-selectin expression, platelet capture of neutrophils, and subsequent homotypic neutrophil adhesion in human whole blood [111].

4.1.2 Polymerization

This section is much shorter than the previous one but it starts with albumin as well. Circulating homo- and hetero-polymeric complexes of albumin show M_r around 210, 168, 147, 132, and 110 kDa and represent between 0. and 2.8% of total albumin [112]. In urine, their presence is an obvious marker of impairment in ultrafiltration selectivity, for instance in association with lupus glomerulonephritis [113]. The surface antigen of hepatitis B virus (HBsAg acts as a receptor for polymerized human serum albumin; its levels are predictive of the outcome of the disease [114,115].

We have mentioned above the main steps of the inhibition operated by alpha-1-antitrypsin on elastase. Pathological mutations, including Glu342Lys (Z), subvert its peculiar conformation to generate a polymerogenic state fo

which at least three models have been proposed. The classical or 'loop-sheet' model involves an intermolecular linkage *via* the reactive loop as a single strand, whereas, in the beta-hairpin model, the intermolecular linkage consists c two beta-strands and, in the triple-strand model, of the three *C*-terminal beta-strands of alpha-1-antitrypsin [99,116,117]. Polymerization occurs mainly at the time of alpha-1-antitrypsin synthesis, with the formation of PAS-positiv inclusions inside the hepatocytes; the resulting pathologically low concentration of the circulating protein (below the protective threshold at ca. 600 mg/L) is unable to prevent proteolytic damage at the sites of inflammation in lungs However, polymers are also found in the circulation of ZZ subjects, both with and without chronic obstructive pulmonary disease (median concentration around 40 mg/L [118]). Polymers may be specifically detected with a monoclona antibody that recognizes a conformation-dependent neoepitope on both polymerized and elastase-complexed molecular forms of alpha-1-antitrypsin [119].

~~4.1.3.~~4.1.3 *Differential glycosylation*

In recent years mass spectrometry, in combination with liquid chromatography or capillary electrophoresis, can reliably assess protein glycosylation, through the analysis of either glycopeptides (after proteolysis) or glycan (after PNGase F or chemical treatment). The evidence provided by these approaches is sharpening our understanding of glycobiology: as exemplified by transferrin [120,121], a protein glycosylation pattern may vary depending on th tissue in which it is synthesized or the biological fluid in which it is accumulated, and is extensively affected by different physiological and pathological states.

Much effort for in-depth investigation of these phenomena is concentrated on pathological tissues. A number of syndromes are being studied in this respect, including multiple sclerosis [122], Alzheimer disease [123] an atherosclerosis [124]. Main focus, however, is on cancer, as glycans are involved in such crucial aspects of the disease as cell signaling and communication, tumor cell dissociation and invasion, cell-matrix interactions, tumo angiogenesis, immune modulation and metastasis formation [125–129].

Most proteins in plasma/serum are glycoproteins; for each of them a number of microheterogeneous species (glycoforms) may be recognized. Systematic changes in glycosylation of plasma/serum proteins are observed i connection with cancer, as exemplified by ovarian carcinoma [130]. Cancer cells produce inflammatory cytokines that influence glycosylation in hepatocytes with an increase in sialyl Lewis^{X}. Sialylation of acute phase proteins increase their half-life and confers *anti*-apoptotic properties thus favoring the survival of cancer cells. Data on glycosylation, *e.g.* the compound influence of branching and fucosylation, may be taken as marker of carcinoma progression an prognosis [131]. A report on experimental mouse cancers detected an increase in internal Neu5Gcalpha2-6 sialylation on the GlcNAc of the Neu5Gc2-3Gal1-3GlcNAc terminal sequence as a common feature but found the substitution c Neu5Gc by Neu5Ac to be induced by colon and not by breast tumor. Among the abundant serum components, transferrin was found to change significantly in terminal glycosylation pattern but not in overall expression level [132].

Glycosylation of plasma/serum proteins is also affected in all types of diseases featuring an inflammatory component [133]. alpha-1-acid glycoprotein (AGP) is the most extensively glycosylated of plasma/serum proteins (ove 50% by weight) and is a positive acute phase reactant, its concentration increasing between 5- and 50-fold in response to noxa. Within the calycin superfamily, lipocalin family, it belongs to the subfamily of immunocalins. Th immunomodulatory and binding properties of alpha-1-acid glycoprotein strongly depend on its carbohydrate composition: strikingly, different glycoforms bring about opposite immunomodulatory effects whereas a pool of *N*-linke oligosaccharide chains attached to a soluble polyacrylamide matrix display a similar activity to its parent AGP in the biological tests [134]. The protein glycosylation pattern is modified by disease: in acute inflammation, a relativ increase of AGP glycoforms with biantennary units is observed (a type I glycosylation change). Conversely, in some chronic inflammatory states there is a relative decrease of AGP glycoforms with biantennary heteroglycans (a type I glycosylation change). This same type of glycosylation changes is also seen in pregnancy, during estrogen administration or upon liver damage [135]. For all these reasons, AGP is regarded as a model and extensively investigated [136].

alpha-1-antitrypsin both inhibits serine proteases and is involved in immunomodulation through effects on neutrophil chemotaxis, immune complex signaling and apoptosis; it appears that the glycans of alpha-1-antitrypsin ar more important for the latter functions rather than for the former [137].

A completely different area of glycobiology to which the analysis of plasma/serum glycoproteins is relevant is the laboratory diagnosis of congenital disorders of glycosylation. For disorders of *N*-glycosylation, causal mutation occur in at least 12 different genes, which encode monosaccharide transferases of the endoplasmic reticulum. Transferrin is often taken as the reference material for the analysis of glycosylation anomalies: by mass resolution, the under glycosylation of transferrin is characterized as the total absence of one or both *N*-linked oligosaccharides [138].

Despite great advancements, the current procedures for direct glycan assessment outlined in the first lines of this section are still complex and lengthy. However, due to the influence of glycosylation on both p*I* and M_r of protein, the quantitation of all the species with 2DE experiments, under conditions allowing their resolution, is a straightforward and efficient approach to differentiate various physiological and pathological conditions.

~~4.2.~~4.2 Inflammation markers

Inflammation is such a broad and general phenomenon that its occurrence cannot be taken as diagnostic of any specific condition. However, its detailed course is finely tuned by the interplay of several factors and some feature vary from one case to another.

The secretion of plasma/serum proteins by the liver follows two main patterns. Proteins such as CRP, serum amyloid A and alpha-1-acid glycoprotein, which require the synergistic action of IL6 and IL1 for maximal induction, ar

said type 1 acute phase reactants (APRs). Proteins such as fibrinogen, haptoglobin, and alpha-2-macroglobulin, which only depend on IL6 for up-regulation, are said type 2 APR; often their synthesis is suppressed rather than enhance by IL1. Some cytokines are selective in inducing one or few APRs; cytokines and other mediators interact in additive, synergistic, co-operative, and antagonistic ways. For instance, as seen in cultured hepatoma cells (HepG2), four c them ~~–~~— IL1, IL6, TNF-alpha and TGF-beta ~~–~~— are required to coordinately induce the synthesis of alpha-1-antichymotrypsin while repressing that of albumin and alpha-fetoprotein. As a result of the above, individual inflammator proteins differ in time-course (rapid- *vs* slow-reacting APRs) as well as in direction and extent of change in their circulating concentration (major positive *vs* moderate positive *vs* negative APRs).

The acute phase proteins function as mediators and inhibitors of inflammation, regulate immune responses, act as transport proteins for products generated during the inflammatory process, and/or play an active role in tissu repair and tissue remodeling. At least some acute phase proteins might constitute an inducible system of factors protecting against cell death by apoptosis: for instance, alpha-1-acid glycoprotein and alpha-1-antitrypsin inhibit th activation of caspase-3 and caspase-7 to become the main effectors (executioners) of apoptosis [139].

Some APRs behave like cytokines: CRP activates macrophages, migration inhibition factor (MIF) prevents chemotaxis. The APRs with *anti*-proteolytic activity block the migration of cells into the lumen of blood vessels an prevent the establishment of a generalized systemic inflammation.

The above summary (taken from the Cytokines & cells online pathfinder encyclopedia (COPE) website at www.copewithcytokines.org) reminds the complexity of the issue. Because they vary during the course of an inflammator condition, group analysis of APRs (serum APR profile) is no doubt more meaningful than measuring any single protein [140]; APR profiles should involve at least one major (CRP or serum amyloid A, SAA), one moderate (haptoglobir alpha-1-acid-glycoprotein, or ceruloplasmin), and one negative APR. Calculation of an index from values of rapid- and slow-reacting positive and negative APRs has been repeatedly proposed in veterinary medicine, because it appears t increase statistical sensitivity and specificity for detecting non-healthy subjects [141].

Examples of disease-specific associations for some APRs are provided by two reports. Inoculation into nude mice of human cancer cell lines derived from stomach, nasopharyngeal, colon, oral and brain cancers results i increased levels of several mouse APRs, including haptoglobin. Conversely, serum amyloid A (SAA), is found only in mice bearing tumors induced by the stomach cancer cell line (SC-M1) [142]. In a genetically modified mouse mode (gp130F/F) that develops a gastric adenoma phenotype, the most acidic isoforms of alpha-1-antitrypsin are down-regulated and the most alkaline are up-regulated in ~~tumour~~tumor-bearing relative to the ~~tumour~~tumor-free cohort [143,144] ~~–~~— this latter case (Fig. 9) being connected as well to the issue of differential glycosylation in Section 4.1.3.

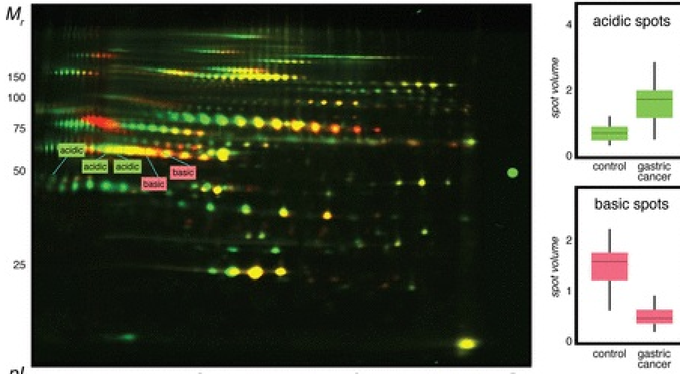

Fig. 9 DIGE of serum from a gastric cancer mouse model, highlighting differentially regulated alpha-1-antitrypsin isoforms; box-and-whisker plots representing the average spot volume associated with alpha-1-antitrypsin in mouse serum, showing up-regulation (top panel, acid spots) and down-regulation (bottom panel, basic spots) in ~~tumour~~tumor-bearing relative to the ~~tumour~~tumor-free cohort. (From [144]).

alt-text: Fig. 9

The highlighted intricacy of cross-influences should suggest the relevance of a detailed investigation of the actual pattern of APRs: in individual clinical cases it could sharpen the diagnostic power of multifactorial information, i individual animal models of disease it could help understanding the mechanisms of disease. A significant factor for the latter is the number and variety of activities each of the APR may be involved in, as sketched in the above an specified in the following.

~~4.3.~~4.3 Moonlighting proteins?

The growing evidence that a single polypeptide chain — a single protein or, better, a single protein species or, when relevant, a single protein domain — can sometimes perform multiple physiologically relevant biochemical o biophysical functions is attracting attention on moonlighting (multifunctional or multitasking) proteins [145]. The ability to moonlight is one of the ways used by cells to carry out many complex processes with a limited number of genes Ancestral moonlighting proteins originally performed a single function, usually enzymatic catalysis, but, through evolution, they have then acquired secondary non-enzymatic roles *e.g.* as receptors, ion channels or chaperones. Som moonlighting proteins can perform both functions simultaneously but others switch roles depending on various factors including the cell type in which they are expressed, their cellular location, the oligomerization status and the bindin of different ligands at different sites. Databases collecting and organizing information about the few hundreds of characterized moonlighting proteins are MultitaskProtDB at http://wallace.uab.es/multitask [146] and MoonPro http://www.moonlightingproteins.org [147]. First attempts have started towards the computational characterization of moonlighting proteins [148] and their identification *via* bioinformatics tools [149].

No plasma/serum protein is listed in the above databases, which only include cellular protein components. Antibodies have been discussed in this perspective to conclude that, while they do moonlight as a class (as they bind t multiple antigen ligands), they do not as individual proteins (since the multiple functions they perform are encoded in different domains) [150]. Ceruloplasmin, on the contrary, in a review article has been acknowledged as moonlighting protein [151]. This plasma/serum glycoprotein is involved in copper transport and storage by binding 6–7 atoms of metal per molecule. There is a question, though, on how the various enzymatic activities the protein is abl to perform (as ferroxidase, ascorbate oxidase, nitric oxide oxidase) are shared between the various metal binding sites. These activities may eventually result in a variety of effects: degradation of organic substrates and defense agains oxidant stress and overall *anti*-oxidant (split word: oxi-dant (see pdf)) activity or, conversely, under peculiar circumstances, pro-oxidant activity.

Without ever being mentioned as such, some plasma/serum proteins actually conform to the definition of moonlighters. For instance, it has long been known that sharply distinct parts of the molecule of alpha-1-acid glycoprotei are relevant either in ligand binding (the inner cavity, or calyx) or immunomodulation (the glycidic chains, see ~~in~~ Section 4.1.3). Other proteins are still becoming associated with previously unrecognized functions. Due to its scattere nature, relevant literature is not easily searched for by keywords; the two examples in the following paragraphs should be taken as anecdotal rather than exhaustive on recent advances.

Acute-phase serum amyloid A isoforms (SAA1, SAA2, and SAA3) are secreted during the acute phase of inflammation. They are known to have several roles, including the transport of cholesterol to the liver for secretion into th bile, the recruitment of immune cells to inflammatory sites, and the induction of enzymes that degrade extracellular matrix. It has recently been demonstrated that human (and mouse) SAAs also bind retinol with nanomolar affinity; th crystal structure of the complex is that of a tetramer with a hydrophobic pocket. During infection, SAAs are up-regulated and their binding retinol *in vivo* contributes to limit the bacterial burden in tissues [152]. Hematopoietic tissu retains pools of stem cells and provides a microenvironment for progenitor cell renewal throughout life. In adults, hematopoiesis is dependent on the chemokine receptor CXCR4 and its ligand CXCL12. An additional role is played b elastase that, when bound to leukocyte surface, acts as a receptor for alpha-1-antitrypsin. Binding of antiprotease to protease forms a motogenic complex [153]. In the blood of HIV-1 patients, alpha-1-antitrypsin (split word: anti-trypsin fo line-break (see pdf)) is bound and inactivated by *anti*-HIV-1 gp120 antibodies; the presence of such immune complexes correlates with the decrease in number of $CD4^+$ lymphocytes in these subjects [154].

In a more general perspective, chaperone-assisted protein folding is one of the processes that concur to proteostasis, i.e. the maintenance of adequate levels of individual proteins in their correct folding state (Fig. 10). We hav mentioned that intracellular chaperoning is one of the additional functions moonlighting proteins are often performing. Recent research has recognized among plasma/serum proteins a growing number of abundant extracellula chaperones constitutively secreted in body fluids, which act as both sensors and disposal mediators for misfolded proteins [155]. The chaperones selectively bind to exposed hydrophobic regions on misfolded proteins to prevent ther from aggregating in the form of toxic insoluble deposits (such as those seen in type II diabetes and Alzheimer's and prion disease). The same chaperones are also implicated in clearing from body fluids the soluble, stabilized misfolde proteins *via* receptor-mediated endocytosis for subsequent lysosomal degradation [156].

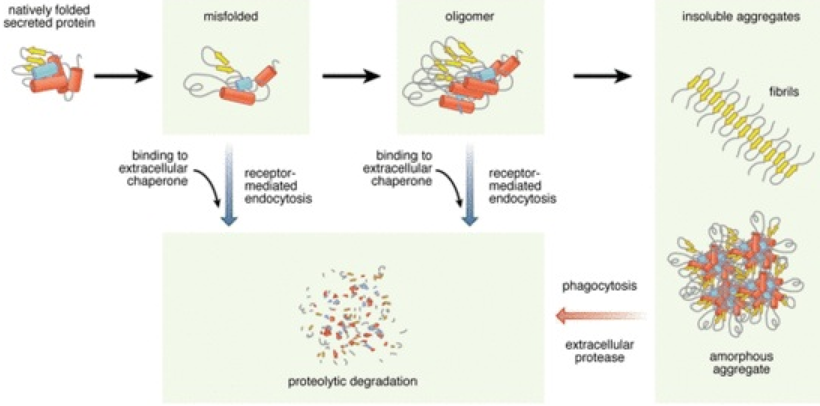

Fig. 10 Major elements of extracellular proteostasis. Proteins undergo rigorous quality control before they are secreted, generally in a natively folded state. Once in the extracellular environment, they encounter a variety of stressors that can cause them to partially unfold and populate misfolded states. Misfolded proteins can aggregate into soluble oligomers and subsequently into insoluble fibrillar or amorphous aggregates. Extracellular chaperones form stable complexes with misfolded protein species, including misfolded monomers and oligomers. These complexes maintain misfolded proteins in solution and facilitate their clearance from extracellular fluids *via* receptor-mediated endocytosis and subsequent degradation in lysosomes. In some cases, misfolded, modified, or aggregated proteins can also be cleared *via* receptor-mediated endocytosis without the involvement of extracellular chaperones; large insoluble aggregates must be phagocytosed. Furthermore, extracellular proteases, such as plasmir may be activated by protein aggregates and subsequently degrade them. (From [155]).

alt-text: Fig. 10

Clusterin, haptoglobin and alpha-2-macroglobulin are the plasma/serum components most directly associated with extracellular chaperone function but individual reports have investigated such property for a number c abundant proteins (apo E isoforms different from the ε4 allele, fibrinogen, alpha-1-acid glycoprotein and lipocalin-type prostaglandin D synthase). Albumin is considerably less efficient at preventing protein aggregation than recognize chaperones but, given its abundance, its activity may be physiologically relevant (e.g. to prevent amyloid formation by Abeta and by the Val30Met mutant of transthyretin).

4.4 Cross-regulation

Extremely reduced levels of individual proteins or their complete absence from the circulation as a result of mutations in the genes coding for some major plasma/serum components are observed as rare recessive disorders. Th pathological consequences each of the conditions entails vary in penetrance and severity. Nature and range of the effects help define the function of the individual protein as well as the redundancy of effectors that may vicariate for th missing component. Moreover, changes in the levels of other components of plasma/serum proteome assess mode and strength of the cross-regulation among protein concentrations. In addition to the analysis of the spontaneousl occurring null phenotypes in the human population, these aspects of proteostasis might be studied in such animal models as the knock-out (KO) mice.

In line with the marginal interest plasma/serum proteins are currently receiving, however, we could find in the literature a satisfactory description of just one genetic disease, analbuminemia, and of just one genetic mode knocking-out of apolipoprotein A-I; only the latter was studied comprehensively with up-to-date proteomics tools. No comparable data have been published *e.g.* regarding the disease that involves the second most abundant plasma/serur protein — atransferrinemia, characterized by abnormal synthesis of transferrin and leading to iron overload and microcytic hypochromic anemia. Nor are reports available regarding alpha-1-antitrypsin deficiency, connected with the (Glu342Lys) or the S mutants (Glu288Val), inherited in the ZZ and SZ genotypes, much more often than with a null-null setup. The most common manifestation of the disorder is emphysema, which becomes evident by the third to fourt decade; a less common manifestation is liver disease, which occurs in children and adults, and may result in cirrhosis and liver failure. In some populations, the frequency of the Z or S genes is especially high, which has been tentativel connected with heterozygote advantage against lung infections.

Analbuminemic individuals manifest mild edema, hypotension and fatigue. The most common biochemical finding is hyperlipidemia, with a significant increase in the total and LDL cholesterol concentrations, but norma concentrations of HDL cholesterol and triglycerides. *In vivo* studies with continuous infusion of labeled amino acids (stable isotope) in analbuminemic subjects detected increased production of IDL, LDL and VLDL and delayed clearanc of VLDL (apoB). Because albumin increases lipoprotein lipase activity by binding free fatty acids, which are strong inhibitors of this enzyme, hypoalbuminemia could be a key factor in the low VLDL clearance. A higher ratio of cholester to TG in apoB-containing particles and an increase in the proportion of cholesterol ester relative to protein could explain the defective catabolism [157]. The findings are similar across species *e.g.* in analbuminemic rats [158]. Overal total plasma/serum protein level is maintained within reference values through the compensatory increase in globulin concentration. Differential regulation is observed; the liver increases at the same time synthesis and secretion of bot positive and negative APRs (of transferrin, hemopexin, ceruloplasmin and fibrinogen and to a lesser extent of alpha-1-antitrypsin, alpha-1-antichymotrypsin, alpha-2-macroglobulin, not of alpha-1-acid glycoprotein) [159] Immunocompetent cells also increase the synthesis of immunoglobulins (IgG, IgA and IgM). Hypercholesterolemia in analbuminemic rats is associated with increased level but unmodified activity of hepatic HMG-CoA reductase, which points to a post-translational regulation of this enzyme and favors an extrahepatic origin of hypercholesterolemia in these animals. Cholesterol 7-alpha-hydroxylase is up-regulated as a compensator response to hypercholesterolemia [160].

An effect on lipid metabolism similar to that in analbuminemia is observed in patients with nephrotic syndrome, in whom the concentration of circulating albumin is lowered by increased loss instead of by reduced synthesis. At difference from analbuminemia, nephrotic syndrome causes severe deficiencies in LDL receptor, HDL receptor, and LCAT. The hepatic response in conditions characterized by reduced plasma colloid osmotic pressure (hypo-oncoti states) involves increased levels of mRNAs encoding a group of secreted proteins, including the negative APPs albumin, transferrin and apo A-I, and the positive APR fibrinogen. Levels of mRNAs encoding negative APRs and fibrinoge correlate with one another, suggesting that they are coordinately controlled. The transcription factors EGRF-1 and HNF-4 seem to be involved in this regulation [161].

Animal models of primary hypertriglyceridemia (apoCIII transgenic mice) and hypercholesterolemia (LDL receptor knockout mice) show higher susceptibility to Ca^{2+}-induced inner mitochondrial membrane permeabilit transition (MPT), a process that can lead to cell death as it is followed by mitochondrial energy failure and the release of pro-apoptotic factors. Both men and mice with analbuminemia, who present high levels of triglycerides an

cholesterol, also present abnormal mitochondrial functions. The search for putative mediators of MPT sensitization in mice revealed a 30% increase in the expression of the apoptotic regulator cyclophilin D together with a 20% decreas in mitochondrial nitrosothiol content: nearly 80% of total plasma reduced thiol groups and nitrosothiols reside in albumin molecules [162].

Our investigation on animals compared mice, in which murine apoA-I had been knocked out, to derived strains made transgenic for human apoA-I, human apoA-II, or both [163] (Fig. 11). The absolute abundance of human apoA- was not influenced by the presence apoA-II, conversely the presence of the apoA-I transgene resulted in a statistically significant increase in expression of the apoA-II transgene. Several proteins not involved in lipid metabolism wer also influenced by the presence of human apolipoprotein transgenes, with apoA-I exerting a much greater effect than apoA-II. All the proteins whose levels are modulated by the expression of human apolipoprotein transgenes — alpha 1-acid glycoprotein, alpha-1-macroglobulin, esterase, haptoglobin and contrapsin — have cytokine-responsive elements in their promoter region and are known to be involved in acute phase reactions. The inhibitory effect of HDL o cytokine secretion [164–166] could be the explanation for our observations on the effect of expression of apolipoprotein transgenes.

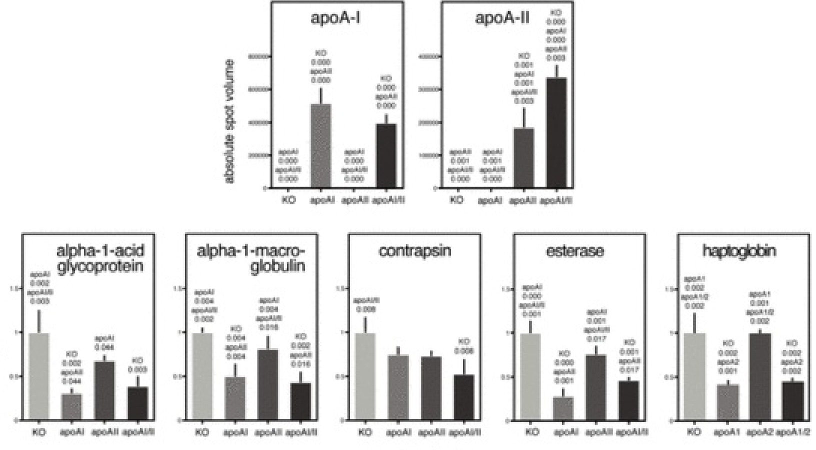

Fig. 11 Variation in protein expression in mice transgenic for human apolipoprotein A-I (apoAI), for human apolipoprotein A-II (apoAII) and for both (apoAI/II), and in their genetic background (mice KO for murine apolipoprotein A-I, KO). (From [163]).

alt-text: Fig. 11

4.5 Immunoglobulins

Immunoglobulins form a large and heterogeneous class of plasma/serum proteins; because of their origin from differentiated B cells of the adaptive immune system (instead of liver) and because of their role as antibodies the deserve a comment on their own in addition to unsystematic mention in previous sections of this review.

Gamma, kappa and lambda-type chains have more alkaline p*I*s than most other major plasma/serum proteins; their removal is relevant for reducing overall protein load more than for preventing spot comigration. The affinity c many Ig classes for protein A or G has been repeatedly exploited for their subtraction; some commercial products feature mixes of resin-immobilized protein A or G with a resin-immobilized triazine dye (e.g. from BioRad) or a resir immobilized *anti*-albumin antibody (e.g. from Biosystems or Sartorius) for the removal of the two most abundant proteins/protein classes in plasma/serum namely albumin and Igs. Immunoglobulin precipitation at 50% ammoniur sulfate saturation is a classical procedure in the production of reagent antibodies, at both laboratory and industry scale [167].

Proteomic procedures have been systematically applied to the field of immunology in order to recognize — in bacteria (e.g. *Neisseria meningitidis* [168] or *Chlamydia trachomatis* [169]), fungi (e.g. *Aspergillus fumigatus* [170] (pluricellular) parasites (e.g. *Amblyomma americanum* [171]), or cancer cells (e.g. prostate cancer [172]) — against which non-self proteins the host organism raises the most intense reaction. This is achieved by separating by 2DE th proteome of the pathogens or some relevant subproteome e.g. membrane proteins, and by using serum from affected patients as a source of primary antibodies in immunoblotting. Proteins in repeatedly detected spots are identifie through MS procedures; acknowledged immunodominant antigens are taken as targets of preventive and/or therapeutic vaccines. This way of proceeding has been given the name of immunoproteomics and focuses on the complement c all identified antigens in a pathogen [173]. Alternative experimental approaches to electrophoresis imply the use of protein arrays of the pathogen.

The same approach is able to recognize transversal and longitudinal variations in the immunoproteome. The latter of course most easily apply to situations in which a pathogen exists in different forms with time such as th alteration of immunoproteome profile of *Echinococcus granulosus* hydatid fluid with progression of cystic echinococcosis [174]. The former allow for the discrimination of more and less severe outcome of a given condition. For instance

the evolution of the infection and the replicative state of the pathogen appear to affect the immunoproteome of *Mycobacterium tuberculosis*, shifting target from membrane-associated to extracellular proteins of the bacillus during th active phase of the disease. Overall, the immune response correlates with bacillary burden but target preferences vary among patients; diagnosis and monitoring treatment outcome may benefit fro~~r~~m the use of the identified biomarker [175]. Finally, *Helicobacter pylori* infection may progress into ulcer or into cancer, a predisposing condition to the latter being atrophic body gastritis. Different investigations have been able to associate *Helicobacter* immunoproteom with the different conditions. Three proteins have been identified as duodenal ulcer-related [176]; sera from atrophic body gastritis and gastric cancer patients differentially recognize 17 *Helicobacter* spots [177].

Through differences in the immune targeting the investigations above exemplify on the interaction between self and non-self during infection/infestation. Tissot and colleagues have evaluated the application of typical proteomi procedures such as 2DE to the diagnostics of lymphoid tissue cancers. A classification of the monoclonal IgGs of multiple myelomas based only on their electrophoretic properties turned out not to be possible because of the extrem diversity among the pathological heavy and light chains [178]. To this purpose, the overall performance of high-resolution electrophoresis was lower than that of zonal electrophoresis with immunofixation. However the opposite is true i selected cases, for instance with the characterization of rare IgD myeloma [179] or with the classification of cryoglobulins [180].

The role of immunoglobulin glycosylation has long been studied [181,182]. As reviewed in [183], current investigation areas include: the glycosylation sites of IgE, IgM, IgD, IgE, IgA, and IgG; how glycans can encode ~~"~~“self~~"~~ identity by functioning as either danger associated molecular patterns or self-associated molecular patterns (SAMPs); the role of glycans as markers of protein integrity and age; how the glycocalyx can dictate the migration pattern c immune cells; and how the combination of Fc *N*-glycans and Ig isotype dictate the effector function of immunoglobulins. Recently, it has been demonstrated that a single glycan on IgE is indispensable for initiation of anaphylaxis [184] In immunoglobulin A nephropathy, galactose-deficient immunoglobulin *A* *(non-Italics)*1 is recognized by unique autoantibodies, resulting in the formation of pathogenic immune complexes that ultimately deposit in the glomerula mesangium and induce renal injury. New approaches using MS have provided insight at pathological IgA1 glycosylation as connected with abnormal expression and activity of several key glycosyltransferase [185].

~~5.~~5 Conclusions

Knowledge of the properties of proteins and understanding of their working is steadily growing; both shared features and individual traits come anew on focus. The interest is currently more on cellula components than on plasma/serum proteome, yet recent advancements ~~–~~— dutifully recorded in this review ~~–~~— have been achieved also on circulating proteins. We maintain that not overlooking information provide by quali- and quantitative changes in major serum/plasma proteins may effectively contribute to our awareness of the cross-influences between various levels of integration in the organism.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2016.04.002.

Acknowledgments

Some of the early achievements of EG and IM have to do with albumin and much of the work in proteomics they share with IE deals with biological fluids. On this background a great deal of the topics covere by this review are linked to our day-by-day experience more than to an assessment of the literature.

Writing our previous review had Mahler symphonies as soundtrack. This time the most often played piece was Händel~~'~~'s Messiah ~~–~~— the number of encore compensating the gloom of the missed liv performance. But, since our interest for music is all but narrow, the title of the review positively comes from a much more recent composition.

~~Bibliography~~References (General comment: on several pages (in the pdf view) the two text columns are not of equal length, e.g. pages 2, 4, 8, 12, 13..., not always for obvious reasons.)

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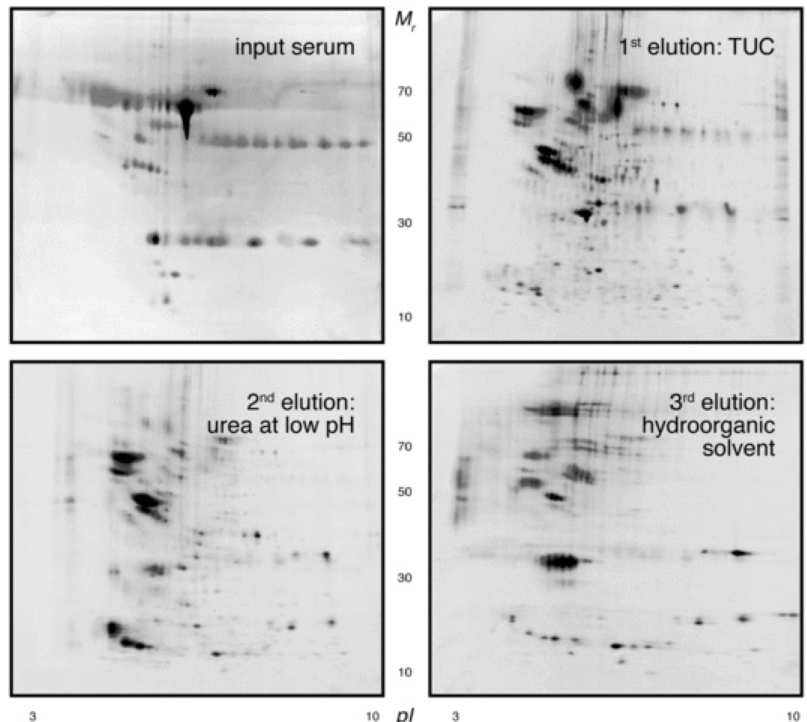
An opposite perspective to depletion of high-abundance proteins from plasma/serum is the enrichment of low-abundance ones through an increase of their relative concentration; such compression of the dynamic range is expected to provide a deeper proteome profiling. This technology was described by the inventors as “protein equalization” and its effect as approaching ~~"~~“democratic proteome~~"~~” [33]. The enrichment procedure is based on a specific and saturable interaction of proteins to a high diversity of binding sites exposed on chromatographic beads. Th combinatorial ligand library is made up of dozens of millions of hexapeptides capable of interacting with most, if not all, proteins in any given proteome [34,35] (Supplementary Figs. 1 and 2). BioRa (http://www.bio-rad.com/) markets the corresponding commercial kit, ProteoMiner, together with 4 elution reagents meant to sequentially recover proteins on the basis of different properties, in a forma compatible with analysis by SELDI. Differential elution under native conditions may be achieved by changing the pH of the buffer [36] or by using a mixture of charged aminoacids (150~~-~~ mM Lys, Arg Asp and Glu) [37]. Elution of all bound proteins under denaturing conditions is made possible by boiling the beads in SDS [38,39]; precipitation with acetonitrile then provides for the highest protei recovery yield and the best 2DE spot pattern [40].

The conceptual background to the peptide ligand library capture is a saturation effect: all the proteins present in the starting sample in concentrations high enough to overload their interactio sites on the beads should be bound in equal amounts, the excess being discarded with the flow-through. After stripping the bound proteins from the resin, the concentration of the higher-abundanc proteins should be equalized in the eluted fraction. Conversely, the proteins present in the starting sample in concentrations too low to overload their interaction sites on the beads should b quantitatively bound. Accordingly, the differences among samples should be intentionally abolished (or at least reduced) for higher-abundance and preserved for lower-abundance proteins. A test of th quantitative performance of the capture beads shows an average variability around 10% in the amount of the isolated proteins, independent of their initial abundance. No normalization effect is in fac

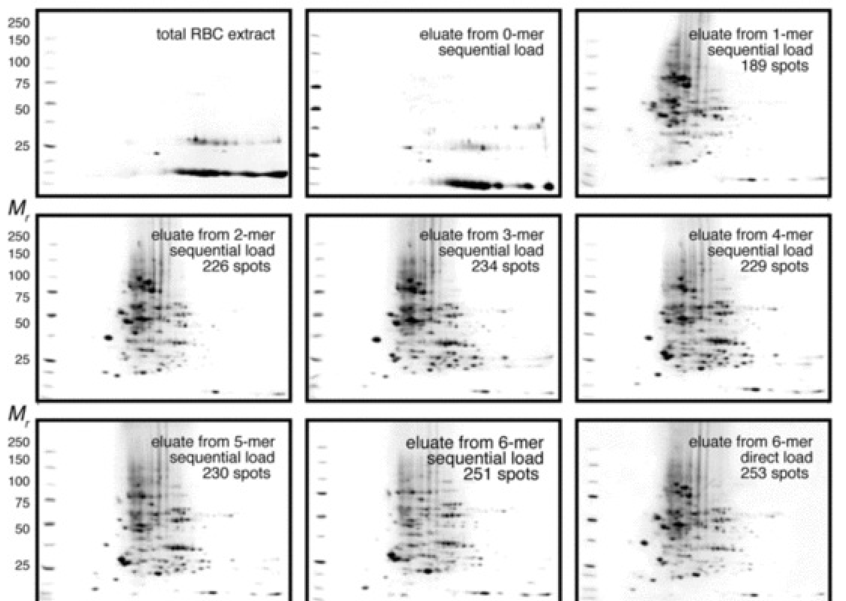
observed but, depending on the protein-to-bead ratio, specific sets of proteins are either enriched or depleted *vs* the starting whole proteome [43] (Supplementary Fig. 3). All reports that evaluate the performance of the protein enrichment strategy [44] or compare it to depletion protocols [45] acknowledge the expected compression of the dynamic range of serum protein concentrations.

The following are the supplementary data related to this article.



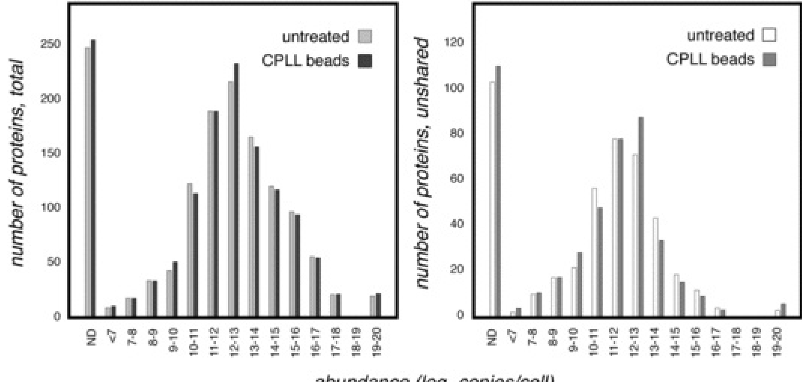
Supplementary Fig. 1 2DE map analysis of control serum (upper left panel) *vs* three ligand library bead eluates. The first elution was accomplished by using three column volumes of TUC solution (2 M thiourea, 7 M urea, 2% CHAPS) followed by the second elution using three volumes of an aqueous solution of 9 M urea, pH 3.5 (pH adjusted with citric acid). A third elution was then performed with a solution composed of acetonitrile, isopropanol, trifluoroacetic acid, and water in the following volumetric proportions: 16.6, 33.3, 0.5, and 49.5%. (From [41]).

alt-text: Supplementary Fig. 1



Supplementary Fig. 2 2DE of eluates from 0-mer (beads without peptides) to 6-mer as a sequence of columns compared to the initial RBC lysate and to the eluate from a hexapeptide column directly loaded with the RBC lysate. The total number of spots counted per map is marked. (From [42]).

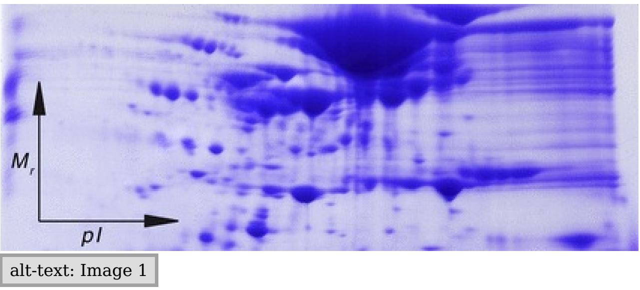
alt-text: Supplementary Fig. 2



Supplementary Fig. 3 Number of proteins identified in samples treated with combinatorial peptide ligand library (CPLL) beads vs the untreated extract, as a function of copies/cell: all proteins (left panel) and unshared proteins (right panel); data for yeast cell proteins (*S. cerevisiae* strain S288C, BY4741). (Redrawn from [43].)

alt-text: Supplementary Fig. 3

Graphical abstract



alt-text: Image 1

Highlights

- The review presents and discusses protocols for depletion of abundant serum proteins.
- It also presents approaches for the effective analysis of whole, untreated serum.
- Next it elaborates on multiple molecular forms-/-multiple functions of serum proteins.
- It then recommends that information on abundant serum proteins be routinely obtained.

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