Elsevier Editorial System(tm) for Journal of Proteomics

Manuscript Draft

Manuscript Number: JPROT-D-17-00362R1

Title: Gender proteomics - I. Which proteins in non-sexual organs

Article Type: SI:Gender Proteomics

Section/Category: Review Article

Keywords: sex; male; female; differential proteins; physiology; pathology

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Graphical Abstract

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Highlights

- The review collects data on the differences in proteome between males and females
- Focus is on our species and both tissues and biological fluids are dealt with
- Stress is on differences under physiological conditions
- Differences related to gender deserve to be assessed in all future investigations

REVIEW ARTICLE

Gender proteomics
I. Which proteins in non-sexual organs

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Table of contents / Index

Abstract

Differences related to gender have long been neglected but recent investigations show that they are widespread and may be recognized with all types of *omics* approaches, both in tissues and in biological fluids. Our review compiles evidence collected with proteomics techniques in our species, mainly focusing on baseline parameters in non-sexual organs in healthy men and women. Data from human specimens had to be replaced with information from other mammals every time invasive procedures of sample procurement were involved.

Significance

As our knowledge, and the methods to build it, get refined, gender differences need to receive more and more attention, as they influence the outcome of all aspects in lifestyle, including diet, exercise and environmental factors. In turn this background modulates a differential susceptibility to some disease, or a different pathogenetic mechanism, depending on gender, and a different response to pharmacological therapy. Preparing this review we meant to raise awareness about the gender issue. We anticipate that more and more often, in the future, separate evaluations will be carried out on male and female subjects as an alternative – and an upgrade – to the current approach of reference and test groups being 'matched for age and sex'.

Highlights

- The review collects data on the differences in proteome between males and females
- Focus is on our species and both tissues and biological fluids are dealt with
- Stress is on differences under physiological conditions
- Differences related to gender deserve to be assessed in all future investigations

1 Introduction

A thorough and systematic assessment of the expression level for the product(s) of each of the protein-coding genes identified in the human genome is being carried out through the construction of a Human Protein Atlas (http://www.proteinatlas.org) as part of the HUPO endeavor (https://www.hupo.org). At the level of organ proteomes, the Atlas database contrasts *testis* and *prostate* to *endometrium*, *ovary* and *placenta*. No data on the contrary are reported about the occurrence of differential regulation of any protein at sites other than gonads and genitals; sex (and age) of tissue donors together with diagnosis are only included in specific histological and Ref-seq data. No sex-specific information is available either from the ProteomicsDB repository, which collects thousands of LC-MS/MS experiments involving human tissues, cell lines and body fluids to provide a draft of the human proteome [1].
Our review aims at filling this gap by compiling evidence on gender-specific differences at the proteomic level throughout organs and tissues. Such differences have been reported for dioichous plants and for many animals in all phyla. We will focus our report on humans and, to a minor extent, on laboratory animals, as their specimens are analyzed under a number of experimental settings as models of physiological and pathological conditions in our species. As we have long ago seen in our investigations [2-4] and as we summarize in Figure 1, the differences between plasma/serum and urine proteomes between males and females in one of these species, *Rattus norvegicus*, are so obvious that the origin of the specimens can be easily guessed from the spot pattern in a 2-DE slab. Subtler yet significant differences are observed in human body fluids and in some human and animal tissues, as we are going to detail in the following.

Our reference list only contains a little more than 100 items. Indeed, on most topics, the reports pointing out gender differences, and proving some features being dependent of sex, amount to a very small fraction of the total of proteomics investigations. In some more cases, mostly dealing with the search for biomarkers of disease, the possible occurrence of gender differences was investigated and eventually ruled out: several features were thus proven independent of sex; to keep our account focused, we do not review these reports but we like to stress that relevance and reliability are equal between evidence in favor or in disfavor of a given conclusion. In most cases, however, the investigated features were made operationally independent of sex through comparison/s between/among sex-matched experimental groups; this was most often the case with difficult to obtain clinical specimens from human patients. This amounts to disregard the influence of sex, if any, and was routine till recently. Indeed, also the endeavor of gender medicine, as aiming at understanding the differences of patho-physiology, clinical signs, prevention and treatment of diseases equally represented in men and women (while not studying either gender-related diseases or diseases prevalent in one gender) [5] is rather recent and incompletely developed. In the past century, biological and chiefly pharmacological investigation in mammals used to be carried out only in males, as the cyclic changes in females connected with their reproductive

function, and the asynchrony of such changes among individuals, was resented as a confounding element. The information collected in this review provides conclusive evidence that such an approach amounts to an unacceptable oversimplification of the biological realm. The limited number of the existing reports, overall and addressing each individual topic; the lack of match for age, genetic background, lifestyle among the reference groups; the extreme variability among the experimental procedures: these are some of the reasons to define the evidence collected so far as a challenge for future endeavor rather than as the end result of past commitment. As we'll see both listing the results in individual areas of investigation and trying to survey the body of present-day knowledge, the state of affairs allows drawing only limited conclusions about recurrent patterns or general trends. This situation urges researchers to go deeper in the assessment of gender proteomics, much beyond the current level of available data, which in many cases are best defined as preliminary.

2 Genes set the scene

2.1 How does it all begin?

In mammals, sex development is a genetically and hormonally controlled process that includes three sequential steps (Jost paradigm [6]). The first such step is the establishment of chromosomal, or genetic, sex (XY or XX) at conception. The second corresponds to gonadal differentiation, towards the formation of either a testis in males or an ovary in females: the former is initiated, at approximately 6 to 7 weeks of human gestation, by the expression of the Y chromosome-linked sex-determining gene called SRY while the latter is the default outcome in the absence of SRY gene products. The third step corresponds to sex differentiation, and involves the development of internal and external reproductive organs and the acquisition of secondary sex characteristics. Male differentiation is controlled by three hormones produced by the testis: Mullerian-inhibiting substance, or anti-Mullerian hormone, testosterone, and insulin-like factor 3; their absence results in female differentiation [7].

Gene SRY encodes a transcription factor that is a member of the high mobility group (HMG)-box family of DNA-binding proteins; because of its function, this protein is named testis-determining factor (TDF). Mutations in the SRY gene give rise to XY females with gonadal dysgenesis (Swyer syndrome); translocation of part of the Y chromosome containing this gene to the X chromosome causes XX male syndrome (http://www.ncbi.nlm.nih.gov/gene/6736). Some hint to the mechanism of action of SRY has been gathered by investigating the effects of its overexpression in two stably transfected lines derived from human testicular embryonic cell carcinoma NT2/D1 cells [8]. Comparing protein amounts by 2-DE demonstrated down-regulation of many chaperone proteins together with up-regulation of laminin, which is important for Sertoli cell differentiation, tubular formation and testis development. Transcriptomic analysis through microarray technology detected higher levels of mRNAs coding for many zinc finger proteins but lower levels for cellular growth factors. Cell growth analysis found inhibition of S or G_2/M transit with arrest of the cell cycle and inhibition

of cellular proliferation. In a different perspective, the first stages of gonadal differentiation in mouse embryos, at the ages of 11.5 and 12.0 days *postcoitum*, were monitored by 2-DE [9]. This extensive survey, carried out by 2D LC-MS/MS, led to the recognition of a few proteins (7 over a total of 1 000) as specific to testis, both adult and embryonic, *vs* other organs – these proteins are likely to have testis-specific roles throughout the life of the organism – and of a larger group (81 for testis and 171 for ovary) as specific to embryonic *vs* adult gonads – these proteins, also expressed in adult organs other than gonads, are likely to have a specific function during organogenesis. In line with the relative quiescence of the ovarian development pathway compared with the morphologically active testis pathway, male samples contained all of the identified proteins whereas female samples contained only a fraction (60%) of them; proteins common to both sexes are likely to have a generic cellular or developmental function within the gonads, whereas proteins uniquely identified in the testis (the remaining 40%) could be involved in regulating embryonic testis differentiation and development. No protein was uniquely identified in the ovary.

2.2 Genetic equity through dosage compensation?

While in the most ancient organisms sex is determined by environmental or social variables, chromosome-based mechanisms are at work in less ancient species, including ours. Through evolution, differentiation of dimorphic sex chromosomes from a pair of autosomes occurred in a number of independent instances, resulting in XY (or X0) systems, with heterogametic males, and in ZW (or Z0) systems, with heterogametic females.
The first event in Y chromosome differentiation in our lineage [10] was a mutation in the SRY-box 3, or SOX3, gene that - not less than 180 million years ago - shifted its function from the regulation of embryonic development to a critical determinant of maleness in the form of the SRY gene. Several internal recombination events (inversions) since caused a rearrangement of gene sequence on the Y chromosome, which restricted recombination with X. Without this mechanism to preserve its integrity, Y became susceptible to deletions, and decreased in size. Despite the transfer from an autosome, some 130 million years ago, of a block of genes (a pseudo-autosomal region that extended the length of both X and Y) *plus* the contribution of four copies of the DAZ spermatogenesis gene, present-day human Y chromosome is only about one-third the length of its X chromosome partner.

To compensate for haploinsufficiency of X-linked genes in males and to restore equal expression between the sexes, dosage-compensation mechanisms evolved, which differ between organisms. In each cell of female mammals a complex and highly coordinated epigenetic process, called X-chromosome inactivation, has been demonstrated to transcriptionally silence one of the two Xs, following either an imprinted or a random pattern; the inactivated chromosome condenses into a compact heterochromatin structure (a Barr body) and is stably maintained in a silent state [11] except, about 15% of the genes in specific regions escape inactivation and an additional 10% are expressed to different extents from some inactive X chromosomes [12]. It was hypothesized that, to compensate the imbalance between monoallelic X-

linked expression *versus* biallelic autosomal expression, in all cells the per-allele expression levels of X-linked genes would be up-regulated 2x (Ohno's hypothesis [13]). The assumed dosage compensation has been investigated both at the transcriptomic and at the proteomic level, leading at first to inconsistent conclusions. Indeed, RNA-seq data in various organs and in different animal species suggested that distributions of gene expression are similar between the X chromosome and the autosomes, except for reproduction-related X-linked genes not expressed in somatic tissues [14]. Instead, by directly comparing mammalian X-linked genes with their one-to-one orthologs in species that diverged before the origin of the mammalian sex chromosomes, both transcriptomic and proteomic data provide evidence for expression halving of X-linked genes during evolution, with the exception of only ~5% of genes that encode members of large protein complexes [15]. An extensive survey (on 2,400 gene products in ten tissues of 5 males and 5 females, via LC/MS on proteins and library sequencing on mRNAs) on embryonic specimens (from *Gallus gallus domesticus*, with heterogametic ZW females) revealed a mean across tissues male-to-female expression ratio of Z-linked genes of 1.32 for proteins and of 1.29 for mRNA. The mean Z chromosome-to-autosome expression ratio was close to 1 in males and lower than 1 in females, consistent with partly reduced Z chromosome expression in females. While these results exclude a general mechanism for chromosome-wide dosage compensation at translation, 30% of all proteins encoded from Z-linked genes showed a significant change in the male-to-female ratio in comparison with the corresponding ratio at the RNA level: some genes showed balanced expression between sexes and some close to 2x higher expression in males [16]. Finally, as summarized in Figure 2, the evaluation of publicly available data for human samples (without sex specification in ProteomicsDB [1]) regarding more than 10,000 autosomal and ca. 300 X-linked genes could find no evidence of X-chromosome dosage compensation at the protein level [17].

The above does not apply to a finite period during preimplantation development: around the blastocyst stage, before X chromosome inactivation, male and female embryos differ in their proteome and in their metabolome as a result of the activities of specific X-linked enzymes and of the effect on the metabolic pathways they regulate. Sex-specific differences in glucose and amino acid utilization have been reported for the mouse and cow blastocysts [18]. Differences are then evident in fetal cells: 2D-DIGE and MALDI-TOF MS analysis detect differential expression between male and female amniocytes for 28 unique proteins (for 5 of which - annexin A1, cathepsin D, cytoskeletal 19, protein disulfide-isomerase, and vimentin – up- or down-regulation exceeds 1.5x [19]).

Sex chromosome abnormalities result in two main pathological conditions. Caryotype 45, X (X monosomy) leads to Turner syndrome, which occurs in about 1 in 2,500 newborn girls, but is much more common among pregnancies that do not survive to term (miscarriages and stillbirths). Caryotype 47, XXY (sex chromosome aneuploidy) leads to Klinefelter syndrome, which affects 1 in 500 to 1,000 newborn boys. Searching the scientific literature we were unable to locate any report on the proteomic features of either tissues or body fluids in the affected individuals. Conversely,

evidence has been collected with the aim of possibly contributing to the diagnosis of pregnancies with affected fetuses. For Turner syndrome, the test sample was maternal plasma, collected during the second trimester of pregnancy. Nine proteins (afamin, C1 esterase, CD5 antigen-like, clusterin, cytochrome c oxidase subunit 3, haptoglobin, hyaluronan-binding protein 2, Ig alpha chain, and sex hormone-binding globulin) were significantly increased in the plasma of women carrying Turner syndrome fetuses, three (kininogen-1, Ig J chain, and transthyretin) were decreased [20]. For Klinefelter syndrome, the test sample was instead amniotic fluid, also collected during the second trimester of pregnancy. Three proteins (alpha-1-antitrypsin, ceruloplasmin, and zinc-alpha-2-glycoprotein) were found at higher levels, four (apolipoprotein A-I, gelsolin, plasma retinol-binding protein, and vitamin D-binding protein) at lower levels in pathological than in control specimens [21].

3 Proteins make the difference

3.1 Can you tell a male from a female from their proteins?

3.1.1 Sex-specific pattern in plasma/serum proteome

Basic information should be regarded as essential; in fact it is often despised as trivial. Along this line, the first proteomic survey on the proteome of male serum *vs* female serum in humans is as recent as 2010 [22].

The first investigation we like to mention, however, is the one by Silliman *et al.*, published two years later [23]: it has a catchy subtitle – “Proteomic analyses of human plasma: *Venus versus Mars*” – and the outline of its experimental plan is straightforward. Five males and five nulliparous females were enrolled; the proteome of their plasma was analyzed after depleting the 14 most abundant proteins by immunoaffinity columns. Aliquots of the treated samples were separated by 1-DE, 10 gel pieces per lane were cut for tryptic digestion, and the resulting peptides were analyzed by LC-MS/MS. This protocol resulted in the identification of a total of 231 proteins; 8 of them were found to differ in abundance between the sexes – six within one order of magnitude, one within two and one within three orders. (A note of caution: The paper does not provide details either on the number of technical replicates or on the statistical treatment; the table summarizing the results does not contain either SD or CV but, for only 2 out of 10 proteins, min-max range.)

Conversely, Miike *et al.* [22] carried out their investigation as a very accurate multistep procedure: 12 males and 12 females were enrolled; the 6 most abundant components were depleted from their serum samples and the proteins fractionated by chromatography on a reversed-phase C18 column (120 fractions/sample); the peptides from trypsin digestion were iTRAQ-labeled and fractionated by chromatography (a strong cation exchanger and a C18-3 column). The final eluent was spotted on a MALDI target (191 spots/fraction); finally, the parent proteins (over 4,000) were identified by MALDI-TOF MS/MS. Less than 8% of the selected hits were > 1.5 times more abundant in female than in male serum, and less than 4% were > 1.5 times more abundant in male than in female serum. The remainder 88% of the

proteins was present in equal amounts in both sex specimens. When arranged according to their function (by MetaCore software), the proteins more abundant in female serum were found to have part in cascades connected with common female diseases, whereas the proteins more abundant in male serum participated in cascades that involved male hormones.

Contrary to the above, the title of the third and last paper on this topic provides little cue to the biological data it contains: "*In silico* instrumental response correction improves precision of label-free proteomics and accuracy of proteomics-based predictive models" [24]. Lyutvinskiy *et al.* describe an *in silico* post-processing method leading to a CV of approx. 1% in the quantitation of >100 abundant plasma proteins after trypsin digestion and LC-MS/MS (apparently without any immunodepletion and/or other preliminary sample treatment/fractionation). The procedure was first applied to pooled plasma samples (from 24 healthy males *versus* 24 healthy females) looking for proteins with the strongest correlation, positive or negative, with sex. When using the 10 best correlating and the 10 best anti-correlating proteins from the pooled sample analysis (data in Figure 3), the model achieved 80% accuracy in sex determinations based on single analyses of individual samples.

How do the three sets of differential proteins in the above reports – collated in Supplementary Table 1 – compare with one another? Well, not much. One obvious reason is the different sample actually analyzed, whether plasma or serum, and whether whole or depleted in a varying number of abundant proteins. As implied by the term depletion (not a synonym for removal, see [25]), it is not surprising that measurable amounts of the targeted proteins can still be found in the processed samples; it sounds however incongruous that a statistically significant difference is observed for the remains of one of the depleted proteins, as it happens for albumin and Igs in [22] and alpha-1-antitrypsin in [23]. In such instances, it would be sensible either to reassess the difference in the undepleted samples, or to remove the hit from the statistics. Uncertainty about the differential concentration of alpha-1-antitrypsin is so extensive that it is reported as higher in females by [23] and as higher in males by [24]. For differential proteins, male/female ratios fall in the range 0.85-1.15 in [22] and 0.25-1.75 in [24] but exceed 100% change in [22]. With sample immunodepletion, the differential items listed in [23] are secretion proteins or are associated to either the exosomes or the plasma membrane; conversely, the items listed in [22] are mainly (intra)cellular proteins. Without sample immunodepletion, all the items listed in [24] are abundant secretion proteins. This includes alpha-2-macroglobulin – which was first reported in 1967 to be higher in women than in men [26] – and albumin – whose age and gender variation was recently surveyed in > 1,000,000 individuals across the UK [27] (see Figure 4). It however does not include other proteins for which there is independent evidence of variation between sexes: for instance apolipoprotein A-I [28, 29] and IgM [30], reported as being higher in females, and IgA and IgD [30], reported as being higher in males. These and other serum proteins for which it is not easy to quote recent bibliography but whose differential levels are known by all clinical biochemists are possibly missed due to inherent bias each procedure knowingly includes.

The main point is of course pretreatment of the sample and chiefly depletion of the most abundant proteins. In addition, effects of charge/size/hydrophobicity can cause some proteins/peptides from a natural mix to go undetected or to become underrepresented *vs* their actual concentration ([31] for 2-DE, [32] for MS): only a concurrence of contributions from various approaches, each addressing a specific set of proteins (a specific subproteome), may provide extensive, and possibly thorough, coverage. In addition to the differences in the type of sample and in the modes of its processing, the investigations we have reviewed make reference to cohorts of very different age – students in [22] and 40-60 year old in [23]; this entails differences in hormonal status within the female group, with inclusion of post-menopausal subjects in the latter but not in the former cohort. Moreover, the enrolled subjects live in different countries (Japan, U.S.A) and thus belong to different ethnical groups, with a different genetic background, and possibly – on average – with different lifestyles and different environmental exposure.

In keeping with the little attention paid to basic information, changes in the proteome of woman serum along the menstrual cycle were investigated in the early '60s but have not been reassessed since with up-to-date procedures. Even the low-resolution, low-sensitivity techniques of decades ago could recognize at mid-cycle definite changes in total protein as well as in concentrations of albumin and globulin fractions [33]: variability of individual components is to be expected as the basis of the above. Indeed, while no systematic investigation has been recently reported on the high-to-medium abundance serum proteins, the circulating levels of a large panel of biomarkers (low-to-very-low-abundance proteins and non-protein compounds, associated with schizophrenia, major depressive disorder, and cancer) were evaluated both in males and in females while taking into account the hormonal status of the latter (cycling, follicular phase; cycling, luteal phase; on oral contraception; post-menopausal) [34]. The concentrations of 5 analytes were found to differ significantly between females in the luteal and follicular phases of the menstrual cycle; that of 26 analytes between postmenopausal females not using hormonal replacement therapy and females with menstrual cycle and of as many as 55 analytes between oral contraceptive users and females with menstrual cycle (Figure 5). To our knowledge, no in-depth investigation with up-to-date procedures has been devoted to the changes in circulating proteins across puberty, in either sex, and across menopause, in women.

3.1.2 Sex-specific pattern in other biological fluids

At a difference from that of other animal species, and notably from that of rats [3, 35], human **urine** is not an easy sample to process for proteomics investigations. From literature data (and our own experience) protein quantitation, mostly in healthy subjects, is highly unreliable for the purpose of balancing sample loads, and protein precipitation is ineffective for the purpose of sample concentration. This is possibly connected with the presence at high concentration of proteins, or better protein fragments, of few kDa in size together with interfering substances [36]. These technical limits add up to be

biological variability in the urinary protein profiles across individuals and between different days of sample collection.
Following careful optimization of the procedures, an in-depth survey by Oh *et al.* on pooled urine samples concluded that the 2-DE patterns are almost identical for men and women [36]. Four proteins, however, were found to be male-specific (5'-AMP-activated protein kinase, beta-2 subunit; cAMP-dependent protein kinase type II-b regulatory chain; tubulin alpha-1 chain; tubulin alpha-6 chain), five to be female-specific (60S acidic ribosomal protein P0; calgranulin; peptidyl-prolyl cis-trans isomerase E; similar to protein phosphatase 1, regulatory subunit 2; transthyretin).
While no list of proteins present in different amounts in male and female samples was released, the conclusions of two more recent papers [37, 38] confirm the above in terms of marginal yet reliable divergence between the proteomic pattern in the two genders, as shown by Figure 6.

Contrary to serum, urine proteins have been analyzed by 2-DE at different stages of the menstrual cycle, specifically mid-cycle phase, luteal phase and after 2 months of contraceptive therapy. A total of 115 protein spots were found to be differentially represented among the subgroups: 40 of them in mid-cycle *vs* luteal phase, 17 in luteal phase *vs* contraceptive therapy, 34 in mid-cycle *vs* contraceptive therapy and 24 in menstrual cycle (irrespective of phase) *vs* contraceptive therapy comparisons [39]. The proteins over-represented in urine after oral contraceptive intake are apolipoprotein J, cystatin A, gelsolin, mannan-binding lectin-associated serine protease 2, S100 calcium-binding protein A9, serpin B3, tetranectin, uromodulin and Zn-alpha2-glycoprotein; the under-represented proteins are serum albumin, aminoacylase 1, fatty acid-binding protein 5, perlecan and S100 calcium-binding protein A8. For many of these proteins a connection is known with the effects of hormonal therapy.

The investigation by Guo *et al.* [38] applies the same experimental scheme resulting in a clear differentiation by hierarchical clustering between male and female urines also to **cerebrospinal fluid** from healthy subjects of both sexes. The proteome of these samples is associated with a lower inter-individual variation than urine (18.4 *vs* 26.2%) and shows no remarkable inter-gender variation in humans. Contrary to our species, differences in cerebrospinal fluid proteome are observed between male and female rats [40], which are, however, less marked than those observed in serum proteome [4] or choroid plexus epithelium transcriptome [40]. As a response to male sex hormone background, four proteins (apolipoprotein A-I, insulin-like growth factor-binding protein 2, or IGFBP2, prostaglandin D_2 synthase, or PGDS, and transthyretin) are more concentrated in the rat CSF samples from females than in those from males. The same proteins are found at higher concentration also in samples from gonadectomized males than in those from sham-operated males; conversely, no differences are observed between sham-operated and ovariectomized females. A few more proteins are found at higher levels solely in females (fructose-bisphosphate aldolase C) or solely in gonadectomized males (transferrin and apolipoprotein E).

Saliva/oral fluids in its unstimulated and acid-stimulated composition has been recently investigated in depth by 4-plex iTRAQ 2D-LC-MS/MS. [41]. In unstimulated saliva 82 proteins, mainly associated with immune function, metabolism and inflammation were found to be gender-specific. On the contrary, no gender-specific proteins were found in acid-stimulated saliva. Sex differences in saliva proteome, however, vary by age group. Fleissig *et al.* consider three such ranges (22–26, 42–46, 78–88 yo). The 2-DE maps of lyophilized specimens contain 300 spots: six of them have higher intensity in young females than in young males (5x for beta-2-microglobulin and/or calgranulin A, 4x for transferrin and/or polymeric immunoglobulin receptor, 3x for calgranulin A and/or Ig J light chain, 2x for leukocyte elastase inhibitor and /or alpha-s1-casein and an unidentified protein). In the male group, eight spots decrease in intensity with age; in the female group, two spots decrease in intensity with age, three spots increase [42]. Sun *et al.* study in three age groups, from children to elderly (5-7, 21-25, 65-90 yo), a specific saliva compartment, the N-glycoproteome; this was addressed by trypsin digesting the pooled concentrated samples, then enriching the glycopeptides via hydrophilic affinity and/or isolating the N-linked glycopeptides via hydrazide chemistry, and finally fractionating and identifying the peptides by LC-MS/MS. In the three test age groups, the number of N-glycoproteins specific to males is 2, 11 and 10, the number of N-glycoproteins specific to females 15, 12 and 6, respectively [43].

Human (reflex) **tears** were analyzed by 1DLC-MS/MS, leading to the identification of 36 proteins: seven of these (alpha-1-antitrypsin, cystatin S, haptoglobin, lacritin, lactoferrrin, lipocalin, mammoglobin B), mainly involved in the local immune defense, were found at higher concentration in female than in male samples [44]. Human **aqueous liquor**, collected during cataract surgery, was processed through 1DE and LC-MS/MS; three proteins were found at higher concentration in female (pigment epithelium-derived factor, alpha-1-antichymotrypsin and plasma protease C1 inhibitor), one in male samples (prostaglandin-H2 D-isomerase) [45].

Evidence about some sex differences in **bronchoalveolar lavage fluid** (BALF) proteome is available for bovines but not for human beings. The concentration of odorant binding protein (OBP) is higher in males than in females under control conditions; following stress, it drops in males but not in females, with a specific decrease of the lower p*I* species [46].

3.1.3 Sex-specific patterns in tissues

It comes to no surprise that the number of reports dealing with sex differences in tissues/organs under control/health conditions is very small, and evidence is limited to animals (mostly laboratory animals, some farm animal).

In the **skeletal muscle** of exercise-naïve mice, 14% of the spots in a 2-DE map (85/608) show significant differences between genders; of these, most of the full-length identified proteins (83%) are more abundant in males, with significant but typically small (>2x) changes; only 5 proteins are more abundant in females (a G protein-coupled receptor, GRP78; the mitochondrial

proteins myoglobin, and electron transferring flavoprotein alpha; the myofibrillar proteins alpha-1 actin and desmin). As seen from the graph in Figure 7, the majority of the differential proteins have metabolic functions: decreased abundance in females applies to all identified enzymes of the glycolytic and to some of the oxidative phosphorylation pathway. Specific concentration differences also involve cytoskeletal and stress proteins; all three phosphorylation states of creatine kinase decrease in abundance in females. No clear preference is observed for the subcellular localization of the differential proteins [47]. In another species, pigs, only one skeletal muscle protein could be confirmed as differentially regulated between males and females (GDP-dissociation inhibitor 1) [48]. Again in mice but in another if related type of tissue, **cardiac muscle**, the number of differential proteins is comparable between sexes, if changing with age (Figure 8); it is also under obvious hormonal control, as at 6 months the number of such proteins changes from 26 (higher in males) / 35 (higher in females) of the intact mice to 26 / 16 of the castrated animals [49]. Most changes are observed for proteins involved in the maintenance of metabolic, transport and developmental processes, as well as for proteins linked to muscle contraction and energy generation; besides changes in overall abundance, in some cases a redistribution occurs among protein species. The different response of male and female murine hearts to phytoestrogen administration is assessed in [50].

Sex-linked differences in mouse **liver** have been investigated both at the transcriptomic and at the proteomic level. The former approach evaluated 14% of the detected transcripts (246/1800) as differentially expressed with 4% at higher levels in females (69/1800) and 10% in males (177/1800). Male rats have a higher expression of genes encoding important proteins for glucose oxidation, glycogen production, lipid synthesis, fatty acid oxidation, and amino acid turnover [51]. Similar findings were reported in another study for the liver proteome of euthyroid and hypothyroid animals (with more changes found in the latter, i.e. 6.7 % of overall spots/protein species changed compared to 3.6 % in euthyroid animals). Authors hypothesized that these physiological differences may explain cases of different susceptibility of female rats towards exposure to lipophilic pollutants [52]. These altered pathways/proteome patterns are in agreement with the finding of a higher metabolic and growth rate and a bigger muscle mass in male rats. Among the female predominant transcripts, fatty acid translocase/CD36 has an exceptional 18x higher mRNA level in female than in male liver; such gender-differentiated expression was confirmed at the protein levels in the rat and at the mRNA level in human specimens [51]. No proteomic data are available for mouse hepatocytes as a whole but for some of their subcellular compartments such as nucleus [53], mitochondria [54] and microsomes [55]; in the latter, most of the cytochrome P450 isoforms have different expression in males and females [56]. Overall proteomic data are instead available for pig liver, in which sex-specific differences affect the levels of 4 proteins (catalase, epoxide hydrolase 1, Hsc70-interacting protein, phenylalanine-4-hydroxylase) [57].
Other reports deal with sex differences in human tissues/organs such as **lung** [58], primary **keratinocytes** [59], and **adipose tissue** [60]; still others with rat tissues/organs such as adipose tissue [61], forebrain ([62] actually, a

comparison between ovariectomy and estrogen replacement) and hypothalamus [63] from **CNS**, and lens [64] and retina [65] from the **eye**.

In the above account we have kept details to a minimum, most often doing without lists of the affected proteins/pathways. Due to the differences in function among the various tissues and organs, the assumption of an identical set of differential proteins seems illogical and evidence demonstrates the hypothesis as untenable except for a very broad tendency of a higher capability towards energy metabolism in males. Conversely, in a few cases an at least conjectural link could be drawn between the proteomic findings and epidemiological data. One of the most interesting reports in this perspective investigates as subtle differences as PTM and their effects on the performance of mitochondrial components [66]. In rat hearts, a higher level in females than in males of the phosphorylation of two enzymes, alpha-ketoglutarate dehydrogenase and aldehyde dehydrogenase 2, brings about opposite effects on their respective activities and synergistic effects on their biological functions: after PTM, the former produces lower amounts of ROS, the latter is more effective at detoxifying ROS-generated aldehyde adducts. These differences are expected to be major contributors to the higher resistance of females to the injuries from the procedures of ischemia/reperfusion in the experimental animals and to the lower risk for cardiovascular disease in human beings.

The finding of differential expression of cytochrome P450 isoforms [56] may be connected with a number of distinctions between males and females: as an example, in female mice, lung microsomes contain higher amounts of CYP1A1 and liver microsomes experience a greater induction of CYP1A2 after hyperoxia exposure; the gender-based female advantage is lost or reversed in Cyp1a1-/- and Cyp1a2-/- animals. Together with a number of other discriminating factors such as inflammatory and oxidative stress markers [67], this may have implications in the sex-specific differences in pulmonary morbidity in humans.

4 Which differences in disease?

Little more than 1% of the proteomics investigations connected with pathological conditions does differentiate test subjects on the basis of gender (https://www.ncbi.nlm.nih.gov/pubmed/). Moreover many of the relevant reports actually deal with animal models of disease rather than with clinical evidence in human patients. Finally, some of the studies do conclude that no significant difference may be observed, in the selected test samples and with the selected experimental approach, between affected males and affected females. On this basis, we have preferred to simply list in Table 1 the references we could locate in the scientific literature and to comment only on few such items.

Table 1 – Gender proteomics in pathology. Differential proteomics findings between affected individuals of either gender

disease groups, by anatomical district or by pathological mechanism	*disease, or disease model*	*references*
cancer	non-small cell lung cancer	[68]
	thyroid cancer	[69]
circulatory system	markers of cardiovascular disease	[70]
	ischemic myocardial infarction	[71]
	cardiac ischemia/reperfusion	(rat model) [66] (mouse model) [72]
	pressure overload	(mouse model) [73, 74]
	cardioplegia	(rabbit model) [75]
	platelets/coagulation	[76-78]
genetic disease	alpha-galactosidase A deficiency (Anderson-Fabry disease)	[79]
	galactosemia	[80]
immune system	HIV	[81] [82]
	HIV resistance	[83, 84]
	Sjögren's syndrome	[85]
metabolic disease	diabetes mellitus	(rat model) [86]
	obesity	[87-90] (mouse model) [91] (rat model) [92-96]
nervous system	Alzheimer disease	[97-99] (mouse model) [100]
	cerebral ischemia/reperfusion	(mouse model) [101, 102] (rat model) [103]
	neuropathic pain	(mouse model) [104]
	schizophrenia	[105]
	trauma	(rat model) [106]
urinary apparatus	stress urinary incontinence	(mouse model) [107, 108]
	renal ischemia/reperfusion	(rat model) [109]

The first investigation we like to single out is the Mayo Clinic proteomic markers of arteriosclerosis study [70] that enrolled at geographically distinct sites over 2,500 men and women of African-American and non-Hispanic White ethnicity belonging to sibships with at least 2 individuals diagnosed with essential hypertension prior to 60 years of age. A panel of 47 protein markers was individually measured in plasma/serum with routine clinical laboratory procedures. Differences were largely consistent across the two ethnic groups and spanned all pathways studied. Female sex was associated with higher levels of several inflammatory biomarkers (C-reactive protein, Hsp27, intercellular adhesion molecule, myeloperoxidase, advanced glycosylation end product-specific receptor, serum amyloid A), apolipoproteins (apoA-I, apoC-III, apoE, Lp(a)), larger LDL particle size, higher levels of adipokines (adiponectin, leptin and resistin), vasodilator peptides (mid-regional pro-atrial natriuretic peptide and mid-regional pro-adrenomedullin plus N-terminal pro-brain-type natriuretic peptide in non-Hispanic Whites), a vasoconstrictor peptide (C-terminal pro-endothelin in non-Hispanic Whites), calcification markers (osteocalcin, osteonectin, and osteoprotegerin, in Afro-Americans)

and thrombotic markers (factors II, V, VII, and VIII, von Willebrand factor, D-dimer, antithrombin III and fibrinogen). Female sex was associated with lower levels of inflammatory markers P-selectin and tissue inhibitor of metalloproteinase-1, lipoprotein-associated phospholipase A2 mass and activity, the vasoconstrictor peptide C-terminal pro-argine vasopressin and the calcification marker osteocalcin.

The second investigation we deem of special interest profiled the whole serum proteomes of age-matched non-diabetic overweight (BMI >25 kg/m^2) and obese (>30 and <35 kg/m^2) females and males. Proteins were resolved in 4 fractions by size-exclusion chromatography and analyzed by iTRAQ 2D-LC-nESI-FTMS. As many as 248 proteins exhibited significantly different concentrations between men and women ($p < 0.05$), which mapped to pathways associated with β-estradiol, lipid and prostanoid metabolism, vitamin D function, immunity/inflammation, and the complement and coagulation cascades [87].

The two investigations have in common the epidemiological relevance of the disease under investigation and the analysis of plasma/serum components, including the major serum proteins. Everything else does differ, as Kim *et al.* perform routine tests for known biomarkers on a large cohort of individuals whereas Al-Daghri *et al.* aim at discovering differential proteomic features. What matters is that either way a number of differences are detected between males and females within the same risk or disease group. In addition to what we have already listed as for the physiological differences in healthy subjects these findings stress the interest in verifying for each condition whether or not all details of a disease are alike across genders – which might imply some consequences at the diagnostic and prognostic levels as well as imply differential requirements for therapeutic interventions.

5 Conclusions

Despite the comparatively low number of investigations devoted to each aspect, there is little doubt that a very high share of proteomic features does differ between male and female subjects, even when disregarding sexual organs. However, in most cases, evidence gathered thus far may only be regarded as preliminary. Heterogeneity in every aspect of the selection criteria and/or of the analytical protocols (some of which we have reported in great detail to stress this point) makes it difficult to compare results from various studies. Indeed, when we tried to find overlaps between lists of differential proteins in different reports, most often we could find none. Supplementary Table 2 collects all available data on differentially abundant proteins in tissues of healthy males and females, as quoted in 3.1.1; the list includes 178 entries and provides, with a uniform/unified layout, identification, function, origin and sex ratio for each of them. Supplementary Table 3 recapitulates the proteins for which multiple hits with identical trend were recorded (at least two entries with male/female ratio either constantly >1 or constantly <1). Only 7 proteins fulfill these criteria: carbonic anhydrase 3; cytochrome P450 2C12, female-specific; cytochrome P450 2C13, male-specific; glutathione S-transferase Mu 1; glutathione S-transferase Mu 2 and phytanoyl-CoA dioxygenase,

peroxisomal, found at different concentrations between males and females twice in rat liver, and L-lactate dehydrogenase, B chain, found at different concentrations between males and females once in mouse skeletal muscle once in rat cardiac muscle. Even when the findings are confirmed by duplicate studies, the quantitative data about sex imbalance vary up to over 20 fold (e.g. for carbonic anhydrase); no overlap between organs/tissue types is obvious.

From Supplementary Table 2 we then extracted functional information about the differential proteins through the collection of GO terms (http://geneontology.org). Supplementary Figure 1 shows the histogram of frequencies for all the terms from all the entries, irrespective of the sample type; Supplementary Figure 2 focuses the information on proteins identified in experiments on whole tissues, as organelle subproteomes are biased by the association with specific functions. Most of the top ranking GO terms in both lists (heme and iron binding, monoxigenase activity, aromatase) describe functions connected with cytochromes, for which a sex-specific expression has been clearly documented; in turn, this implies a differential handling by tissues, and chiefly by liver, of all compounds, endogenous as well as exogenous, and is the basis for the more and more often acknowledged difference between men and women of the responses to both therapeutic treatments [110] and toxicological noxae [111]. Specifically, aromatase is the enzyme (estrogen synthetase; EC 1.14.14.1) that catalyses the synthesis of estrogens from androgens (demethylation of androgens' carbon 19 with production of phenolic 18-carbon estrogens), influencing the physiological balance between the sex steroid hormones. The enzyme is present, with tissue-specific isoforms, not only in the gonads (and in placenta) but also in brain, adipose tissue and bone; its targeted expression is controlled by a complex mechanism involving alternative promoter utilization [112]. Aromatase is known for its roles in reproduction and in reproductive system diseases (especially as a target for inhibitor therapy in estrogen-sensitive conditions). However, besides reproductive and homeostatic actions, estrogens influence cell cycle, metabolism, immunity, vasculature functions, brain development and performance, and bone remodelling. All of these aspects have a counterpat in differences between males and females in baseline indicators of health conditions as well as in disease susceptibility [113].

As an effect of the enormous variability among the procedures, the large share of potential biomarkers supposedly identified by each of the quoted reports contrasts with an extremely limited number of actually validated biomarkers. It may only be hoped that the scientific community prioritize the topic of gender-related differences in order to eventually move from the exploratory to the confirmatory phase. Although we use here the term *biomarker*, we mean it in the broader sense of indication of a specific condition, whether physiological or pathological. In our opinion, a clear definition of the differences between males and females, under baseline conditions, both in tissues and in biological fluids, is not for curiosity but for understanding the basics of biology in our species. To emphasize the risks of neglecting to take into account sex-related differences we like to quote the conclusions by Ramsey *et al*. [34] on their work on biomarkers, which shows

that as many as 96 proteins differ between men and women and, in addition, in women, 5 change along the menstrual cycle and 26 between pre-and post-menopausal phases. On a technical perspective, Ramsey *et al*. stress that failure of matching cases and controls for sex and hormonal status during a proteomic investigation results in an unacceptably high false discovery rate, which may peak up to one order of magnitude above the outcome with properly matched groups. On a biological perspective, Ramsey *et al*. remark that the observed variance provides a rationale for the differences in disease susceptibility across the human population depending on sex and hormonal status.

Our closing considerations go to the need for top quality investigations in a global proteomic perspective that encompasses both high and low abundance components. In our opinion biological fluids should be given the highest priority because of their relevance both in routine procedures and in research protocols. As we had mentioned in the above sections, no global investigation presently exists assessing serum composition either along the menstrual cycle or across menarche and menopause.

For work on non-pathological tissues it is much more difficult to have access to human than to animal samples, and even the latter are all but trivial material. For this reason, the information obtained by processing such specimens should be pushed at the highest possible level. Both sexes should be tested – and tested separately. Also pre/post-puberty and, for women, pre/post menopause groups should be clearly divided, in order to take into account the effects of the hormonal status of the subjects. Very much is to be done to go beyond preliminary data: it should definitely be done in the next future.

Acknowledgments

We have listed at length our proteomic ‘ingredients’. A nursery rhyme has an alternative answer.

What are little boys made of?	What are little girls made of?
What are little boys made of?	What are little girls made of?
Snips and snails	Sugar and spice
And puppy-dogs' tails	And everything nice
That's what little boys are made of	That's what little girls are made of

(Early 19th century; Roud Folk Song Index number = 821)

Figure legends

Figure 1 – 2-DE pattern of serum and urine from male (left) and female rats (right) as resolved on a 4-10 NL IPG and on a 7.5-17.5% T PAA gradient (modified from Figure 3 in [4]). The names of the proteins for which differences are systematically observed between genders are marked; the increase falls below 2-fold in most cases but amounts to orders of magnitude for thiostatin in female serum and for major urinary protein in male urine [2, 3].

Figure 2 - Mean values across 17 non-sex-specific human tissues of the ratio between gene products from the X chromosome (305 genes, having excluded those known to escape from inactivation in females) and from all the autosomes (10,735 genes for which both transcriptomic and proteomic data are available). From left to right: mRNA concentration; protein concentration; protein concentration relative to mRNA concentration. The test tissues were: adipocytes, adrenal gland, bone, colon, gall bladder, heart, kidney, liver, lymph node, pancreas, salivary gland, skin, spleen, stomach, thyroid; all analyzed data refer to human samples, without sex specification, and are publicly available in ProteomicsDB [1]). Selected data redrawn from Figure 1 in [17].

Figure 3 - Male/female concentration ratios for plasma proteins. Drawn from the values in the rightmost column of Table 1 in [24], set of individual LC-MS/MS data, *in silico* corrected for the variability of the instrumental response.

Figure 4 – Median serum albumin concentrations stratified into age groups of five years and male and female gender. Redrawn from data in Table 1 in [27].

Figure 5 – Top: Plot of the first two principal components from PCA on concentration data for a panel of serum biomarkers; color-coding according to sex and female hormonal status, see legend. The percentage of variation accounted for by each principal component is shown in brackets with the axis label. Modified from Figure 1 in [34]. Bottom: Number of differential biomarkers, according to sex and hormonal status, as marked. The inset inside the male *vs* female column specifies the number of biomarkers found at higher concentration either in males or in females. Drawn from in-text data in [34].

Figure 6 – Results from LC-MS/MS on 10 male and 10 female urine samples plus 1 pooled male urine sample (P). A: Comparison of average protein/peptide overlap rate from intra-run, intra-gender, and inter-gender urine analyses. B: Heatmap of the overlap rate between each sample pair. C: Hierarchical clustering of the samples. Redrawn from Figures 3 and 6 of [37].

Figure 7 – Grouping according to gender, function and subcellular location of the differential full-length proteins between exercise-naïve male and female murine biceps brachii. Left panel: percentage of proteins at higher concentration in males (blue) or in females (red). Redrawn from in-text data and Figure 2 of [47].

Figure 8 – Differential proteins in male (blue) and female (red) mice depending on age. Drawn from data in Table 1 of [49].

Supplementary Figure 1 - All proteins in Supplementary Table 2 were grouped by GO-Molecular Function. Entries were then classified in 2 categories: either M>F or F<M. Only groups with a significant proportion of entries with concordant ratios according to binomial test were retained.

Supplementary Figure 2 – Same as for Supplementary Figure 1, making reference only to experiments carried out on whole tissues.

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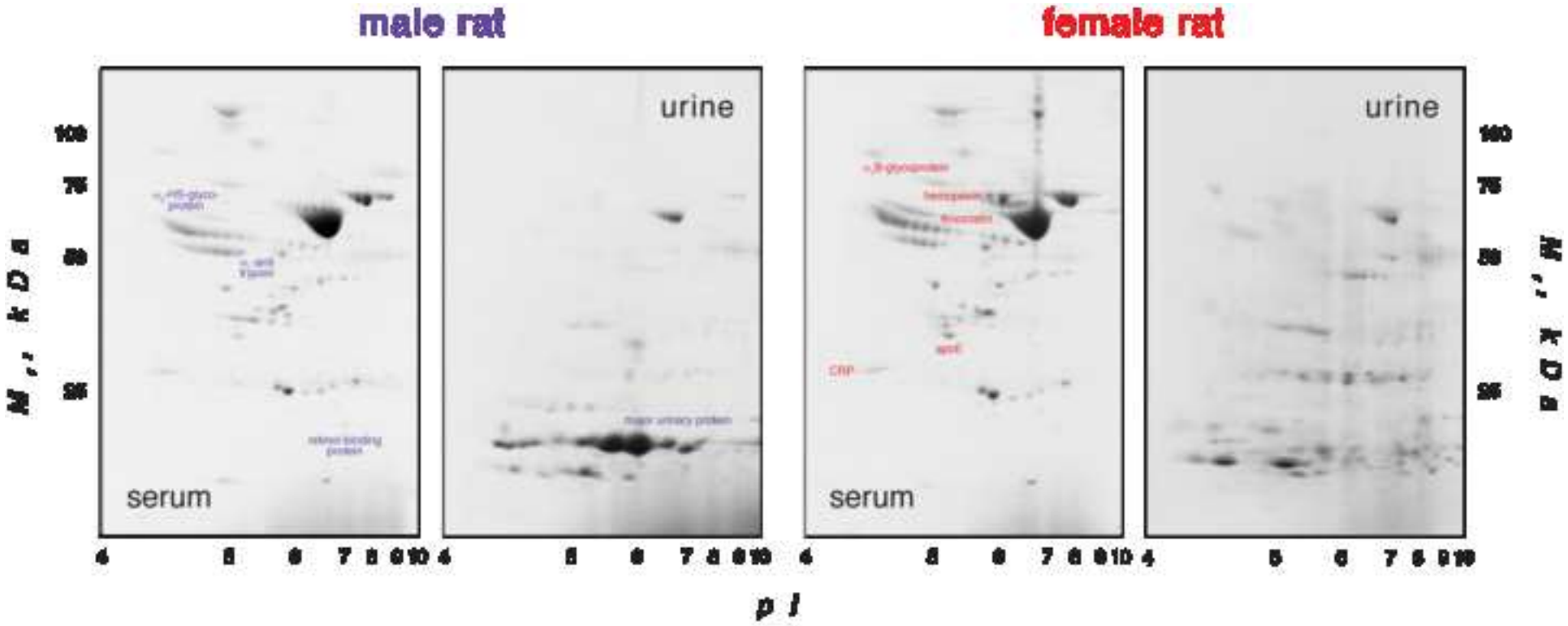

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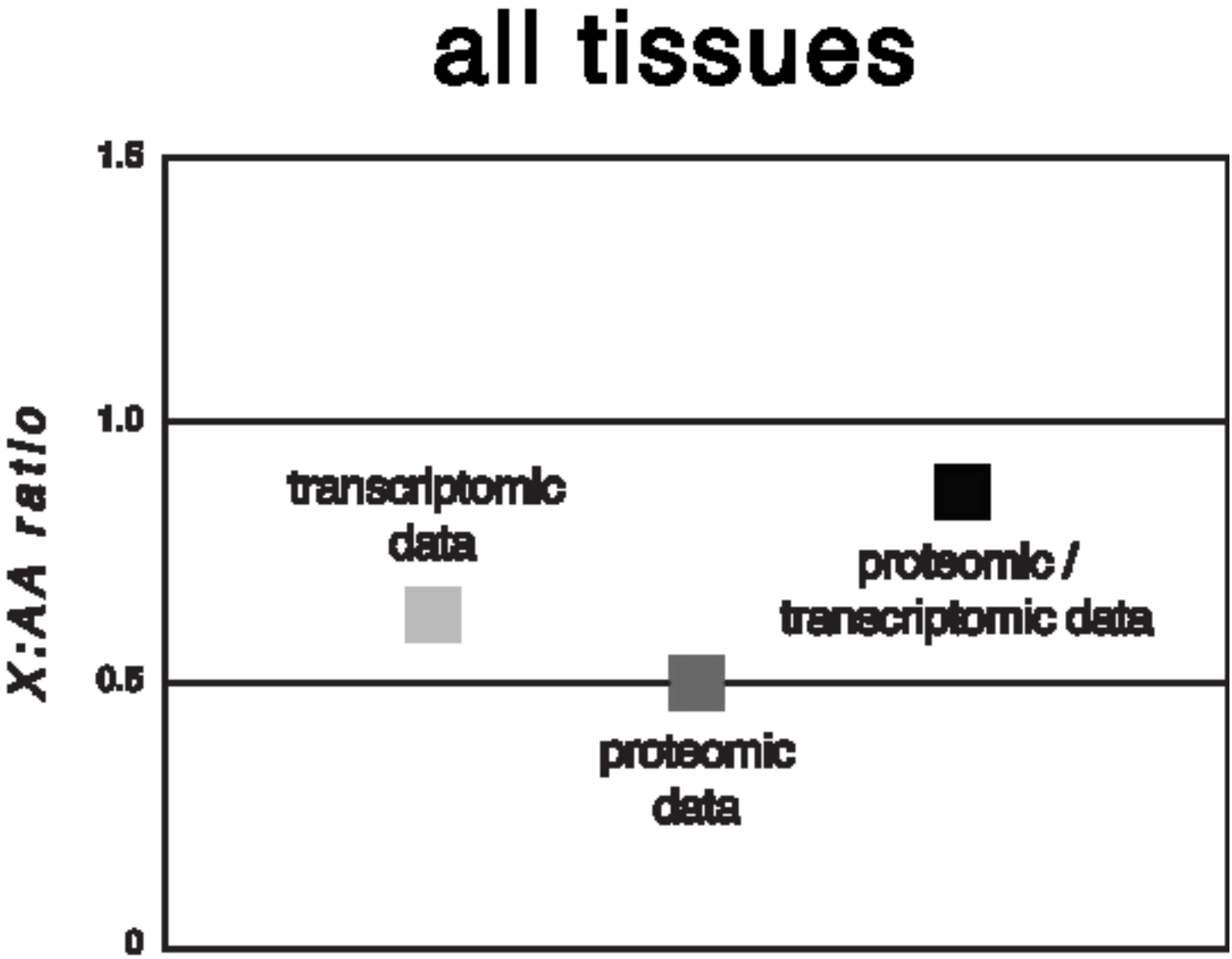

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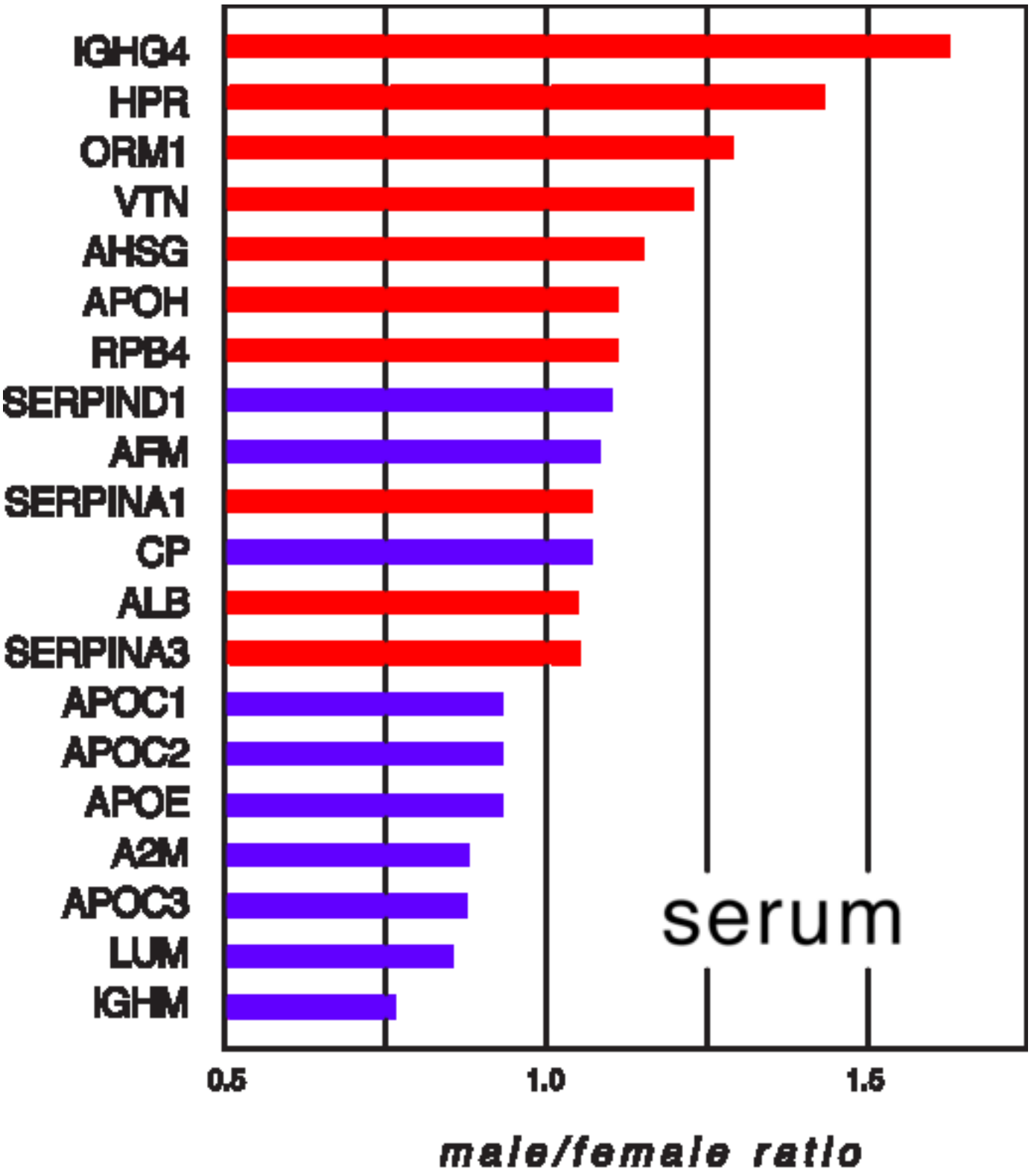

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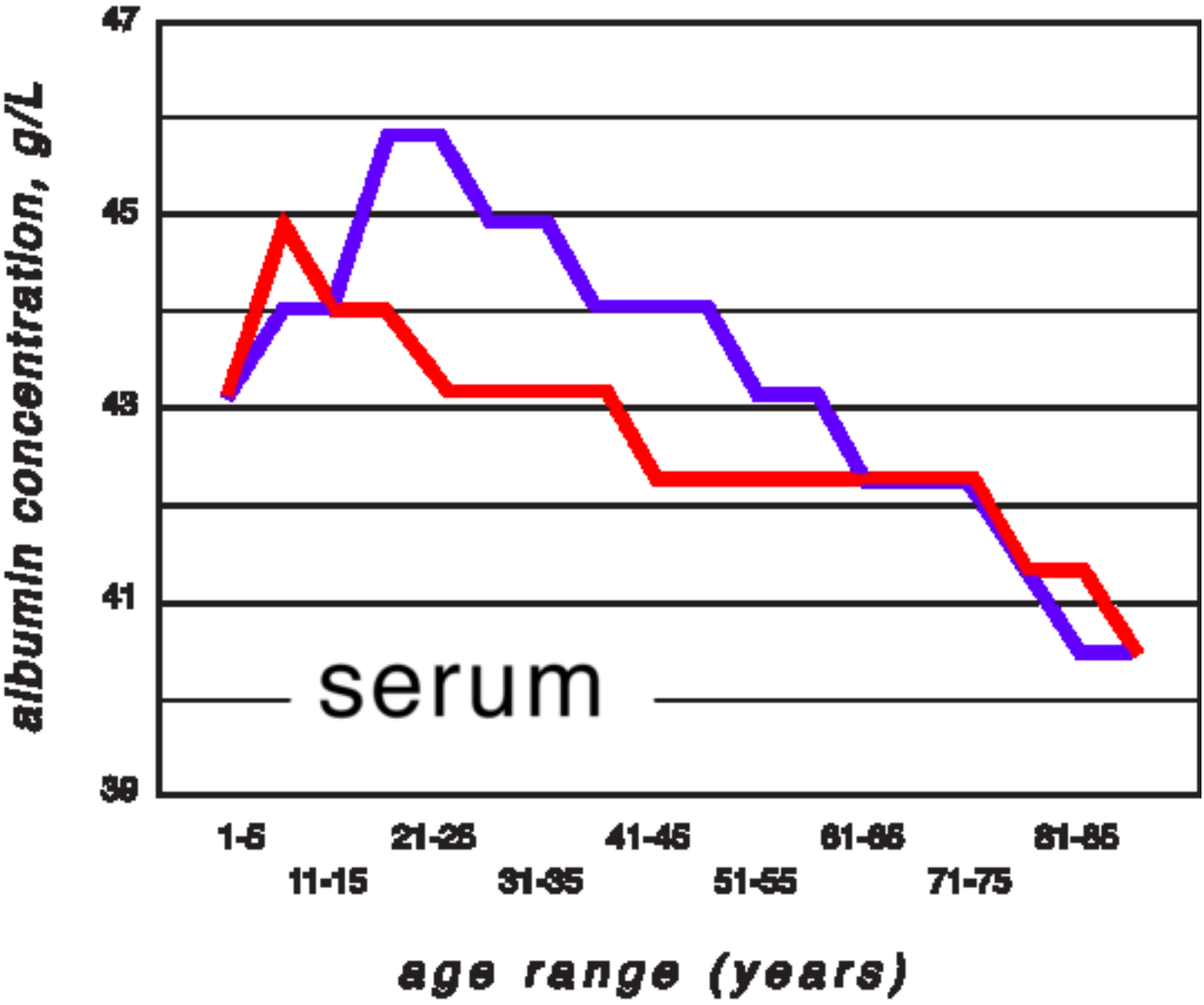

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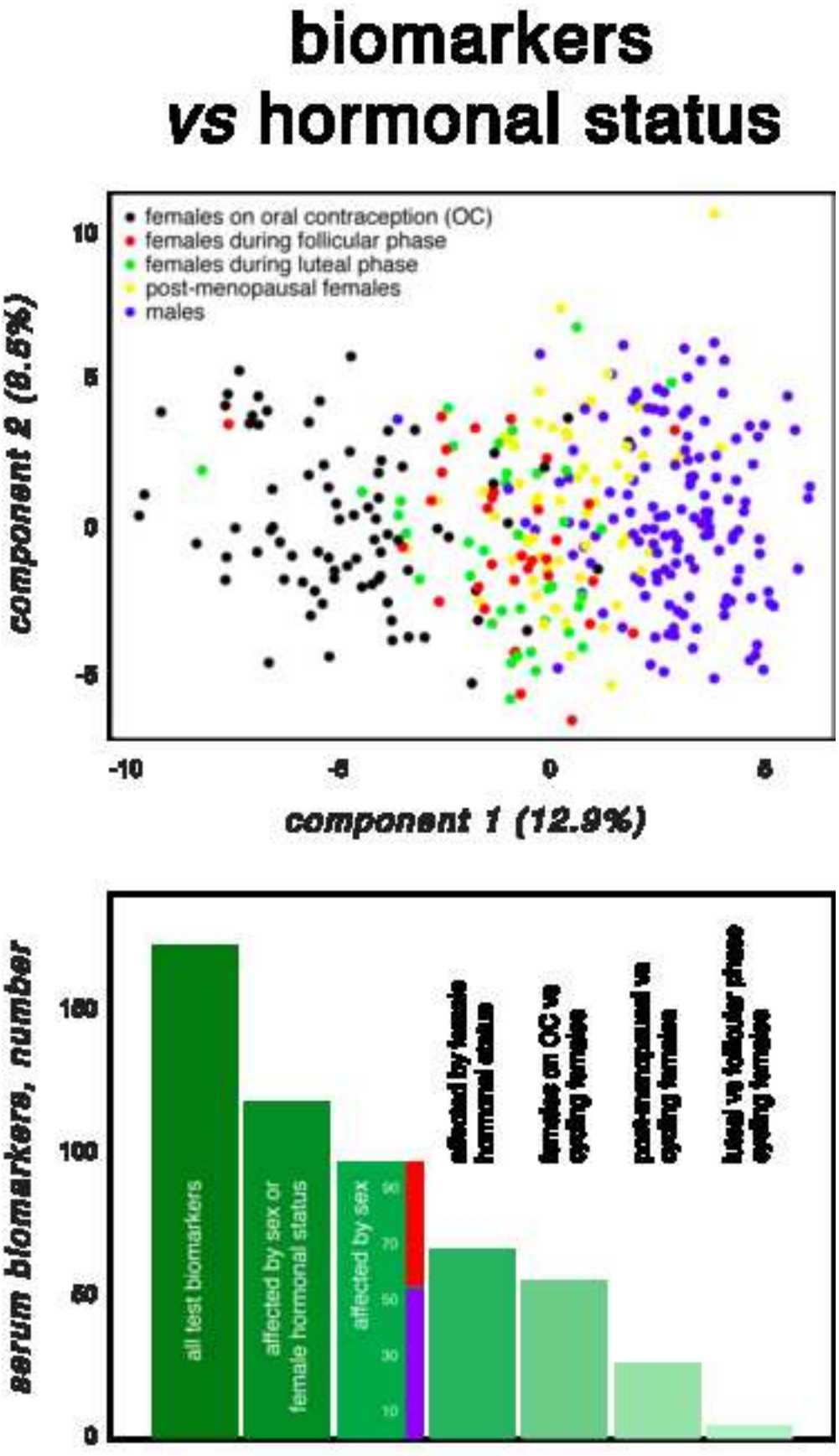

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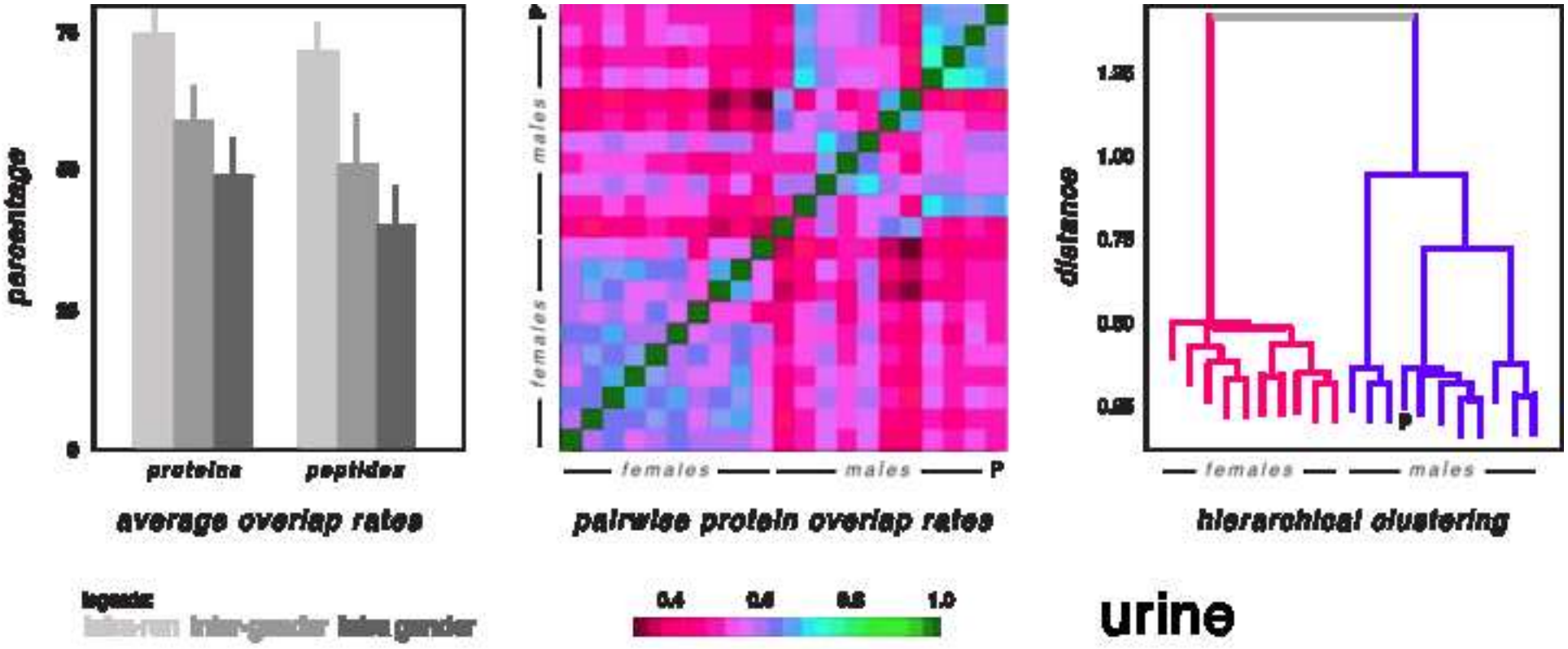

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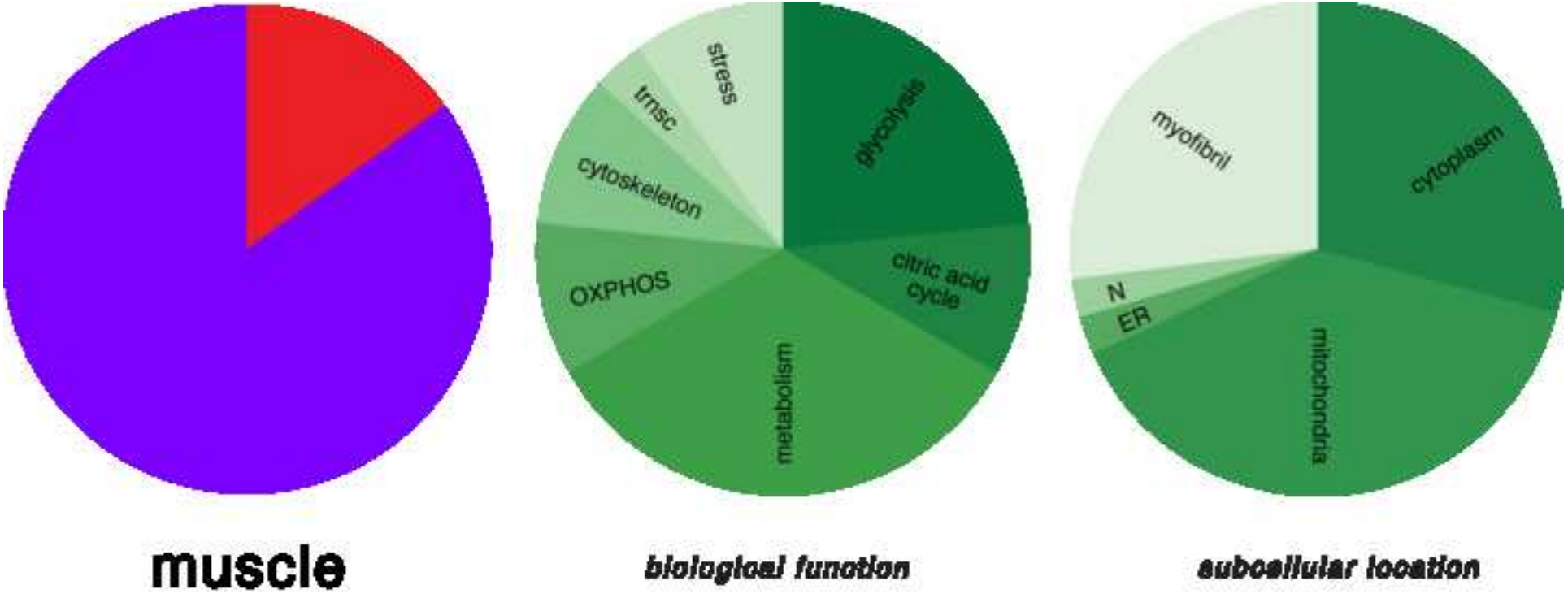

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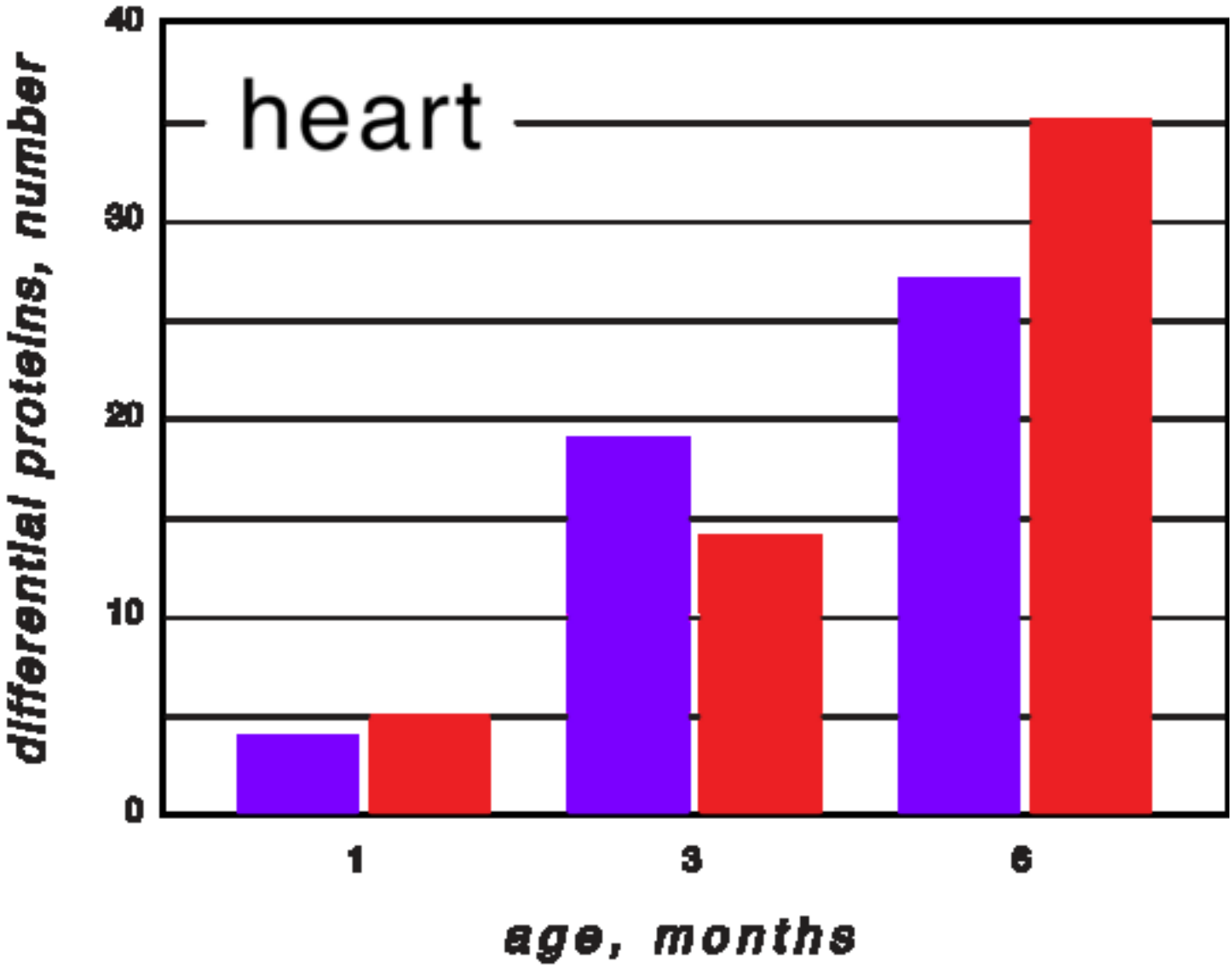

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