



# Serum and Plasma Proteomics for High Altitude Related Biomarker Discovery

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## Abstract

Serum and plasma are widely used biological fluids for a large variety of biochemical and pathological tests which have been used since historical times for diagnosis and prophylactic analysis. High altitude physiology and related pathologies are geographically localized socio-economic issues of a large set of population. In the last few decades, considerable research has been done to explore the underlying proteomic changes in serum and plasma of humans as well as in the model organisms. Recent studies have led to the emergence of a number of potential prophylactic and therapeutic targets which enable better and timely diagnosis, effective therapy, and also susceptibility testing of high altitude related illness. As proteome is highly dynamic in time and space, and serum or plasma is known to reflect the proteomic changes in various organs. Although analysis of plasma or serum is an invasive approach, yet the specificity of plasma-based markers holds great promise in diagnostics. In this chapter, we will discuss general approaches to use plasma and serum proteome, followed by examples of potential biomarkers or indicators of hypobaric-hypoxia-induced perturbations.

## Keywords

Serum · Plasma · Biomarker · High altitude pathophysiology · Hypobaric hypoxia

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## 9.1 Introduction

Plasma proteomics has been a cornerstone of biomarker discovery for a large number of physiological and pathological conditions since historical times. The use of plasma proteins for the diagnosis and assessment of various organ function tests is popular enough across clinicians. Although diseases and pathological conditions show less wide alteration in the proteins compared to altered physiological conditions, hence it is challenging to establish a bio-molecular marker in physiological conditions compared to the pathological condition. High altitude physiology or hypobaric hypoxia is a less-known yet complex physiological condition that is characterized by alterations in the proteomic profile of various organs mediated by physiological responses. A large number of proteomic studies including basic studies using 2D gel electrophoresis or differential gel electrophoresis (DIGE) were performed in the past in the authors' lab and were later re-established using newer and more reliable approaches including targeted and quantitative proteomics. With the increasing availability of proteome spectra database and improved mass resolution in mass spectrometry, the number of proteins identified per sample has substantially increased and thus brought about the refinement of the proteomic alteration during high altitude exposure. While the key question of proteomics studies in high altitude remains elucidation of physiological malfunctions under hypobaric hypoxia, the exact chronology of molecular events and physiological changes is yet to be established. Nevertheless, adaptability to high altitudes especially by comparing natives and lowlander travelers is yet another prospective area under investigation. Defense establishments have an advantage in gathering organized study subjects due to homogenous age-matched and diet-matched individuals, which is otherwise not possible to attain. In more than a dozen studies, thousands of differentially expressed proteins have already been deciphered to follow a pattern and therefore with a potential to be established as a biomarker either for high altitude sickness susceptibility or indicators of hypobaric-hypoxia-adaptability. This chapter mainly focuses on various proteomic approaches using plasma/serum as samples. Some of the studies by authors and other potential biomarkers are highlighted in the end.

## 9.2 Plasma and Serum and Their Potentials in the Diagnosis

Serum and plasma both are blood fractions in liquid form which are free from any type of cells. However, the serum is obtained after clotting the blood, hence it is free from clotting factors, while plasma is obtained from unclotted blood (by collecting blood in an anticoagulant medium) and hence richer in terms of proteins. The protein concentration in plasma/serum is approximately 60–80 mg/mL and around 50–60% proportion is occupied by some of the most abundant proteins, namely albumins and 40% globulins, in particular, 10–20% are immunoglobulin G, the most prominent immunoglobulin (Leeman et al. 2018), rest 40–50% are other proteins, some of which have very low abundance. In the early days when proteomics approaches were

not advanced enough, it was difficult to study the low abundance proteins and enumerate the diversity of plasma proteins, but now with the help of high-resolution mass spectrometry (HR-MS), several studies have independently enumerated the profiling of serum/plasma proteins to about 15,000, encoded by more than 12,000 genes (Nedelkov et al. 2005). Present-day proteomics methods also provide techniques for sequestering and thus eliminating the high abundance proteins by affinity-based approaches making the identification of low abundance proteins easier. Besides the canonical plasma and serum proteins, which play roles in hemodynamics and other plasma functions, several other non-canonical proteins are often secreted from various organs and therefore reveal the pathophysiological state of various organs. In fact, the use of isoenzymes, limited to specific organs, has been historically used for organ function tests. More recently, the availability of high throughput quantitative proteomics approaches, such as iTRAQ, MRM, TMT, etc. has provided opportunities to researchers to explore the minute differences in protein levels in various physiological or pathological conditions. These advancements have certainly empowered the proteomic biomarker discovery pipelines and several prospective proteins are increasingly being described as potential biomarkers for pathological or even physiological alterations. Plasma or serum is considered a good alternative for tissue or biopsy sampling for proteomic studies, especially for human studies, due to their less invasive nature; however, other samples such as urine or saliva are completely non-invasive but low diversity and low abundance of proteins in those samples is still a challenge and keeps the importance of plasma and serum relevant in the pathophysiological context.

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### 9.3 Proteomics Approaches to Study Plasma and Serum

Serum preparation requires the collection of blood and separation of serum by placing the blood-containing tube in the ice for an hour after collection for coagulation of blood (coagulation can be hastened by adding agents like thrombin), followed by centrifugation. The supernatant after the centrifuge contains serum proteins. Plasma separation involves the addition of coagulants, e.g. EDTA, heparin followed by centrifugation. Serum and plasma contain several abundant proteins such as albumin and immunoglobulin, which sometimes need to be removed using commercially available affinity columns if the study is aimed to investigate lesser abundant proteins.

Isolation of the proteins from serum and plasma begins with cell disruption, e.g. sonication, followed by precipitation, and finally, purification to remove even the slightest impurities, for proteomics. There are several methods for the precipitation of proteins such as the use of cosmotropic agents (urea) which breaks the hydrogen bond between molecules and denature it, chaotropic agents (ammonium sulfate) which promote the formation of water–water hydrogen bonds, and protein losses hydration and precipitates out, a phenomenon known as salting out. Ionic precipitation methods of precipitation use acids like trichloroacetic acid, salicylic acid. Proteins can also be precipitated by organic solvents such as acetone, ethanol,

etc. based on their potential to dehydrate and remove the water of hydration. After precipitation of protein, removal of salts and other impurities needs to be achieved through methods such as ion-exchange chromatography or dialysis, before downstream proteomic processes. One must take precautions in sample collection, handling, and storage of the samples and standardization of the proteome extraction method; otherwise, it can affect the reproducibility of the results (Blonder et al. 2008). Several proteomics approaches are available, such as gel-based including one-dimensional and 2D polyacrylamide gel electrophoresis, and gel-free including label-free and labeled such as isotope-coded affinity tag (ICAT), isobaric tag for relative and absolute quantification (iTRAQ) shotgun proteomics, etc.

### 9.3.1 Immunoblotting

Immunoblotting is a rapid assay for the detection of target proteins that works by exploiting specificity inherent in antigen–antibody recognition. The step involves electrophoretic separation of proteins, followed by transfer and bindings to the nitrocellulose/nylon/PVDF membrane, treatment with selective primary and a secondary antibody, and visualization using chromogenic or chemiluminescent substrates (Gallagher and Chakavarti 2008). Immunoblotting has been one of the most conventional proteomic approaches in understanding effect in the high altitudes (Gangwar et al. 2020; Lopez et al. 1975).

### 9.3.2 2D Gel Electrophoresis and Mass Spectrometry

2D polyacrylamide gel electrophoresis is a widely used technique to separate and visualize the proteins based on their mass and charge (Aslam et al. 2017) followed by identification of selected protein spots using mass spectrometry (MS). In the 2D gel electrophoresis, the proteins are separated into two steps, first in the one dimension using the pI values and then in the second dimension based on their relative molecular weight. Upon comparison of gels from different experimental conditions, selected proteins are generally chosen for further identification through MS. MS measures the mass-to-charge ratio, thus determining the molecular weight of the proteins. MS involves three steps: the first step is to transform the peptides into gas-phase ions. The most common ionization methods include MALDI (matrix-assisted laser desorption ionization), SELDI (surface-enhanced laser desorption/ionization), and ESI (electrospray ionization). The second step is the separation of ions on the basis of mass/charge ( $m/z$ ) values in the presence of an electric/magnetic field and the final step is to measure the  $m/z$  values of each separated ion (Aslam et al. 2017). 2D gel electrophoresis and MS have provided insights into the human proteome changes at high altitude conditions (Ahmad et al. 2013).

### 9.3.3 Non-gel Based Quantitative Proteomics

Quantification of proteins in a sample can be mainly performed by 2D gel electrophoresis or MS. MS can identify and quantify the changes in the protein (Matthiesen and Bunkenborg 2013). In general, quantification can be done either by labeling peptides or can be label-free. Among the labeling, the most common ones are isotope-coded affinity tag (ICAT), isobaric tag for relative and absolute quantification (iTRAQ), stable isotope labeling by amino acids in cell culture (SILAC), isotope-coded protein labeling (ICPL). These labels are useful to study protein changes in complex samples (Veenstra 2007). The development of the label-free approach has helped achieve faster, cleaner, and simple quantification results. In this technique, different proteome samples are prepared and separated separately using LC-MS/MS or LC/LC-MS/MS followed by protein quantification. Quantification is based on two categories, first, measurement of ion intensity changes (such as peptide peak/peak heights) in chromatography, and second is based on spectral counts for individual samples. Direct comparison between different sample analyses tells about changes in the protein abundance (Zhu et al. 2010). MS-based proteomics in high altitude stress and diseases have been carried out extensively in the last one decades on both humans and experimental animals under different conditions such as hypoxia (Gao et al. 2017).

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## 9.4 Key Considerations for Biomarker Discovery for High Altitude Physiology

Biomarker discovery has been a long pursued domain of clinical sciences which has got attention with the advent of omics technologies. A typical dictionary definition of a biomarker is “a naturally occurring molecule, gene, or characteristic by which a particular pathological or physiological process, disease, etc. can be identified” or “a biomarker that may predict aggressive disease recurrence in liver transplant recipients” (Oxford dictionary). The standard pipeline of biomarker discovery using proteomics includes several important steps. The first and foremost consideration is about the sample choice and experimental regimens. Simulated hypoxia on experimental rats might not be physiologically identical to the actual hypobaric hypoxia observed on high altitudes. Also, the physiological responses of humans at a specific altitude are different from experimental animals such as rats. Hence, conclusions from simulated hypoxia on animal models may not be directly extrapolated for the biomarker discovery. It is therefore important that separate experimental regimens and models must be chosen to specifically define hypoxia markers.

The next important aspect in using proteomics for biomarker discovery is the nature of the proteomic approach. Classical approaches such as 2D-gel electrophoresis are now considered obsolete and non-reproducible, hence potential biomarkers identified using 2DGE must be validated enough to be used clinically or confirmed using alternative sensitive and reproducible methods. Emerging approaches such as

quantitative proteomics (using iTRAQ, TMT, or label-free) have been known to show a better coverage and hence preferred over conventional mass spectrometric methods. Moreover, targeted proteomics and shotgun proteomics are also emerging approaches for biomarker discovery.

Another highly important consideration for the biomarker discovery in high altitude biology is the choice of sample. Most often plasma or serum is considered as a good choice due to its richness and easily identifiable secretory proteome of various organs. However, one must consider depleting highly abundant proteins such as albumin and immunoglobulins to reach out proteins of low abundance and overcome their masking effects. Several commercial kits and methods are routinely practiced during serum/plasma proteomics. Besides plasma and serum other biological fluids have been utilized for biomarker discovery with limited success.

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## 9.5 Potentials Biomarker Candidates in High Altitude Pathophysiology

Several proteomics studies have already been conducted over several decades across various laboratories on humans at high altitudes or in contained experimental hypoxic models using hypobaric chambers. These studies have reported several potential biomarkers based on proteomics studies. Studies by Sharma et al. using 2D gel electrophoresis reported SULT1A1 as a potential biomarker associated with gradual changes in the pulmonary proteome of those exposed to hypobaric hypoxia. These studies were conducted on rats at a simulated altitude of 7600 m, approximating some of the highest mountain peaks in the greater Himalayas (Ahmad et al. 2015). Ahmad and Sharma also performed extensive studies on plasma and serum proteome profiling of rats and humans in independent studies and demonstrated the upregulation of several proteins including vitamin D-binding protein, hemopexin, alpha-1-antitrypsin, haptoglobin  $\beta$ -chain, apolipoprotein A1, transthyretin, and hemoglobin beta chain while downregulation of transferrin, complement C3, serum amyloid, complement component 4A, and plasma retinol-binding protein (Ahmad et al. 2013). In similar studies on rats, hypobaric hypoxia-induced changes in several proteins including Ttr, Prdx-2, Gpx-3, Apo A-I, Hp, Apo-E were recorded (Ahmad et al. 2014). In yet another study by Sharma et al., plasma proteomics of high altitude pulmonary edema (HAPE) patients, a few proteins, namely acute phase proteins (APPs), complement components, and apolipoproteins among others. Among the APPs, haptoglobin  $\alpha$ 2 chain, haptoglobin  $\beta$  chain, transthyretin, and plasma retinol-binding precursor were found to be differentially expressed indicating their potential for being developed as a biomarker (Ahmad et al. 2011). Brain, which is also the most-affected organ due to hypobaric hypoxia, was also evaluated for proteomics changes in experimental conditions and it was observed that glycolytic enzymes like Gapdh, Pgam1, Eno1, and malate-aspartate shuttle enzymes Mdh1 and Got1 in the cortex as compared to hippocampus deciphering efficient use of energy-producing substrates. This was coupled with a concomitant increase in the expression of antioxidant enzymes like Sod1, Sod2, and

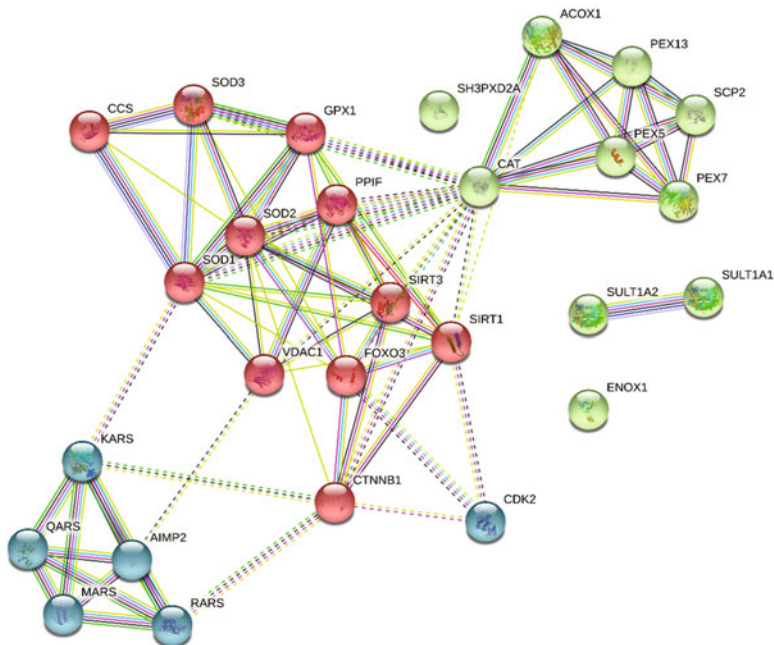
Pebp1 in the cortex (Ahmad et al. 2011). In yet another study by Gayatri et al., 2D gel electrophoresis-based proteomic analysis has revealed proteomics markers for hypoxia susceptibility in experimental rats. They reported upregulation of several antioxidant proteins, namely TTR, GPx-3, PON1, Rab-3D, CLC11, CRP, and Hp in hypoxia tolerant rats, while apolipoprotein A-I (APOA1) was upregulated in hypoxia susceptible rats. Furthermore, proteomics analysis of Ladakhi natives using 2DGE followed by MALDI-TOF showed functional regulation between the renin-angiotensin system and eNOS-cGMP pathway and concomitant elevation in the levels of eNOS, phosphorylated eNOS (Ser1177), and plasma biomarkers for nitric oxide (NO) production (nitrate and nitrite) as well as the availability of cGMP (Padhy et al. 2017). 2D electrophoresis is not considered a highly accurate technique at present due to non-reproducibility issues (Magdeldin et al. 2014) and hence establishing the differentially regulated proteins in the aforementioned studies remains a challenge. Furthermore, with the advancement in the proteomics approaches, recent studies were performed using state-of-the-art proteomics approaches such as iTRAQ based quantitative proteomics and tandem mass tag-based quantitative proteomics. More recently in a study by Pooja et al., tandem mass tags (TMT) based proteomics showed elevated plasma concentration of apolipoproteins APOB, APOCI, APOCIII, APOE, and APOL, and carbonic anhydrases (CA1 and CA2) during hypoxia exposure which was also corroborated with lipid profiling suggesting a potential perturbation in lipid transport and lipoprotein-associated metabolic and molecular pathways (Pooja et al. 2021). These studies with advanced proteomic approaches suggest that identification of a single proteomic marker is barely possible for such a complex physiological perturbation and hence a larger focus should lie on understanding and proposing entire biochemical pathways or protein networks. Some of the common biological processes which can be potential hubs of biomarker discovery for hypobaric hypoxia-induced pathology or adaptation are elaborated in the following text.

### 9.5.1 Antioxidant Signaling

One of the most pertinent hubs of protein interactions observed across the aforementioned proteomic studies was the effect on redox milieu and antioxidant signaling. Several highly interacting proteins such as superoxide dismutase, sulfotransferase, thioredoxin, glutathione peroxidase are often shown to be altered during hypoxic insult in both experimental animals and humans as evident from several past studies. Antioxidant signaling is therefore on the key event in controlling (Fig. 9.1).

### 9.5.2 Lipid Metabolism

Several proteomic and lipidomic studies conducted on high altitude dwelling individuals or high altitude travelers have indicated a significantly changed lipid



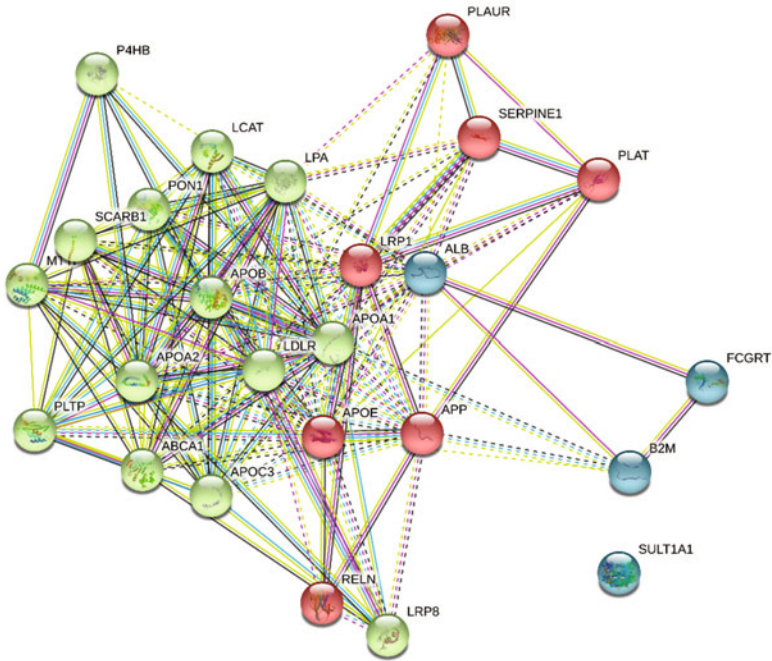
**Fig. 9.1** Biological network of potential redox regulating proteins known to show altered expression in hypobaric hypoxia with a potential to be used as biomarkers (K-means clustering, FDR <1%)

profile and lipid regulated pathways perturbations, among which albumin is known to play a nodal role. Albumin, which acts as a carrier of several lipids post-lipogenesis in adipose tissues, is mostly held accountable for hypoxia-induced edema. Concurrently, proteins interacting with albumin are among the potential biomarker candidates which form yet central nodes in hypoxia-perturbed proteomic networks (Fig. 9.1).

### 9.5.3 Cytoskeleton Remodeling

More recently, studies conducted by Paul et al. observed that an interplay of lung cytoskeletal elements exists that helps in achieving redox homeostasis and extended survival in hypoxic environments. Qualitative perturbations to cytoskeletal stability and innate immunity/inflammation were also observed during extended low  $pO_2$  exposure in humans exposed to 14,000 ft. for 7, 14, and 21 days (Paul et al. 2021). However, to date, scanty information is available for presuming it as a potential biomarker and thus further studies are required to be conducted in a more extended manner to identify potential makers from the cytoskeletal remodeling during hypoxia.





**Fig. 9.2** Biological network of potential lipid metabolism-related proteins with albumin occupying a nodal place and hence suggesting a highly orchestrated interplay in hypoxia-mediated changes in lipid metabolism

### 9.5.4 Post-Translational Modifications

Post-translational modifications induced by hypoxia have recently gained the attention of researchers, especially those mediated by redox signaling. Among the prominent hypoxia-induced protein modifications are carbonylation and nitrosylation. Due to their wide occurrence and diversity in pattern, carbonylation and nitrosylation are possibly the next generation biomarkers for hypoxia-induced stress at the proteomic level. Shotgun and targeted proteomics approaches using biotin switches and DNPH pulldown are often used for the analysis of these modifications. In a study by Anamika et al., the relative carbonylation and nitrosylation in hypoxia-induced samples have been studied with proteome-wide mass spectrometry studies and potential sites of these modifications have been highlighted (Gangwar et al. 2022) and suggested a direct and indirect interaction between nitrosylation and carbonylation pertaining to blood-coagulation and inflammation networks provoked by redox signaling.

## 9.6 Future Prospects and Conclusion

The future of serum and plasma-based biomarker discovery for high altitude pathology and adaptability is promising. With over several decades of significant scientific contribution and rapid advancements in the proteomic sciences, much clarity on proteomic perturbations has been achieved, yet we are steps away from an exact identification of proteomic markers. In fact, the pathology of high altitude-related changes is so complex that it is very unlikely that a single proteomic marker would be sufficient and achievable for diagnosis or prognosis. However, it is suggested that large-scale proteomic screens or panels with multiplexing features including the expression analysis as well as a post-translational modification would be highly valuable for diagnosis and screening.

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