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Research Article

Exosome: A Model to Study the Protein-Protein Interaction in Biofluids

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Received: March 18, 2021; **Published:** May 27, 2021

DOI: 10.31080/ecop.2021.12.00773

Abstract

Protein-Protein interactions are vital for physiology and their quantitation is crucial for pathology, as most of the functional proteins exert their functions through protein interactions. Protein networking in the body fluids plays a role in homeostasis and thus is a valuable tool for disease diagnosis and drug targeting. Biofluids may also serve an early biomarker, since change in the proteome of biofluids is prior to the clinical manifestation. The bottleneck is the presence of high abundant proteins in biofluids which hampers the detection of low abundant proteins. An alternative approach can be the isolation of exosome which represents an ensemble of tissues and cells. Hence, we did an *in silico* analysis of the exosome proteome and total proteome of the biofluids. Our analysis showed that 51.7% of salivary, 86.2% of plasma and 43.64% of urinary exosome proteome are representative of the respective total proteome. Thus, the exosomes can be a miniscule of the total proteome devoid of high abundant proteins. The percentage of common proteins between total and exosomal biofluid proteome, among the plasma, saliva and urine was found to be 3.68% and 2.4% in the respectively. Our study on the protein-protein interaction of exosome proteome using STRING10 has found exosomal proteins to be interconnected, forming functional nodules. Thus, we propose that exosomes can be an ideal model to study protein-protein interactions in biofluids.

Keywords: *Protein-Protein Interactions; Exosomes; Mass Spectrometry; In Silico*

Introduction

The sequencing of complete human genome gives us the information of 30,000 genes (International Human Genome Sequencing Consortium 2004 [1]. The genome sequence provides a blueprint yet the utility by a cell depends on its type, and cellular environment. The mapping of complete human proteome seems unattainable and one of the reasons is post translational modifications which yield different forms of a protein under a defined condition. For e.g. AMPK (Activated protein kinase) and phospho AMPK (Thr172). Protein functions and activities are often regulated by other proteins and thus they do not work alone. Protein-protein interactions (PPIs) play a critical role in physiology and pathology through a shift in kinetics of equilibrium. Such kinetic shift results in the formation of a new binding site, activation or inactivation of proteins, and brings change in the substrate specificity of proteins. PPI can be of two types - (i) Transient interactions (ii) Stable interactions. Transient interactions are weak, temporary and require phosphorylation, glycosylation etc. Stable

interactions are strong physical interactions like receptor-ligand binding. Since proteins interact dynamically with specific partners for the biological functioning [2], information on the interacting partner reveals its functional role.

The total proteome can be classified as (i) secretory and (ii) non-secretory. Under physiological conditions, secretory proteins are secreted on to the biofluids (plasma, saliva, urine etc). The protein concentration in biofluids is found to be either elevated or depleted in pathological conditions [3]. Biofluids represent an ensemble of tissues, and those which can be collected non-invasively (saliva and urine) are attractive sources for biomarker discovery [4]. Different biofluids present different challenges, however plasma and urine are considered to be more complex among them [5]. There are 10,000 and 1,200 proteins reported in plasma and saliva till date. The challenge lies in cataloguing the total proteome due to several reasons. One such reason is the presence of high abundance proteins at a concentration of mg/ml [6,7] which limits the detection of low abundance proteins in ng-pg/ml concentration. To overcome this, high abundance proteins are depleted by fractionation or the protein of interest is enriched through Immunoprecipitation (IP). Hence, there is a possibility of losing the proteins bound to abundant proteins [8] while urine, a product of blood filtration has less protein concentration. The protein concentration of a normal person is less than 100 mg/L, while the total urine output is 1.5 L/day. Hence normal protein excretion is less than 150 mg/day, which is 1000 times less than that of plasma. Thus, studying PPI in biofluids is very challenging. To the best of our knowledge, there are very few reports of PPI in biofluids (saliva, plasma, urine). The noted methods available for studying PPI include the following: Pull down assay, Yeast two hybrid assay, Surface plasma Resonance. The techniques compatible for biofluids are Co-Immunoprecipitation followed by Mass Spectrometry (Subtractive proteomic approach) and Crosslinking Protein Interaction Analysis (TAP). Newer approaches like, microscale thermophoresis are also being used to study PPI in biofluids [9]. However, high individual variability in the concentration (Urine collected in the morning contains more proteins than in afternoon and evening) and state of modification of plasma proteins make the PPI study even more challenging [10]. Thus, the knowledge on PPI in biofluids still remains elusive. Hence the need of the hour is to identify representatives of biofluids (plasma, saliva, urine) as models for PPI.

Exosomes are naturally produced membrane vesicles of around 30 - 100 nm size, released actively by cells [11]. They are also found in biofluids including the plasma, urine, saliva, malignant effusions, synovial fluid, breast milk, bronchoalveolar lavage fluid and epididymal fluid [12]. Exosomes carry DNA, RNA, lipid and proteins to the target cells [10]. ExoCarta, an Exosome database has reported that exosomes contain 9,769 proteins (www.exocarta.org). Exosomes are considered as the future of biomedicine due to their diverse applications such as use as biomarkers, therapeutic targets and natural nanocarriers [13]. Comprehensive proteomic and lipidomic analyses of exosomes derived from prostate cancer cell line have provided insight both as a biomarker and a therapeutic drug carrier [14]. The presence of Prostate cancer biomarkers (PCA-3 and TMPRSS2) in the urinary exosomes of prostate cancer patients [11] has intrigued researchers to focus on exosomes from biofluids. Exosomes have also been used to deliver anti-cancer drug in hepatocellular cancer cell line [12]. Zhuang., *et al*. demonstrated the potential of exosome as a carrier in LPS-challenged mice [15] to cross the blood brain barrier to reach brain microglial cell when delivered intranasally. In spite of its remarkable applications in biology and medicine, some questions in the field of Exosome biology remain unanswered like, Are exosomes specific? How does the host cell recognise the exosome to be taken up? How do exosomes specifically select proteins and RNA to be transported out from the host cell? Do those proteins and RNA have specific signal sequences which facilitate their release via exosomes? Can cell-specific exosomes be isolated from biofluids, plasma, saliva, urine? These are some of the open questions to be answered in the near future. In this article, we report an interesting observation through in silico validation and analysis on exosomes which may play a possible role in mediating the transport of proteins from cells to biofluids. Our study has found that the proteome of exosomes are specific to the source of their origin and destination where the proteins present in exosomes have potential to interact with each other. Further validation with experimental data will strengthen the possibility of using exosomes as an ideal model to study PPI in biofluids (plasma, urine, saliva) surpassing the current challenges in PPI study for researchers.

Objective of the Study

The objective was to identify a model to study PPI in biofluids (plasma, saliva, urine), which will provide information on biofluids. The presence of exosomes in biofluids makes it an alternative of biofluid. The key questions we have addressed through this study are, (i) What is the role of exosomes in biofluids? (ii) Are exosomes specific to particular organ? (iii) Can PPI occur in exosomes?

Methodology

Enumeration of the total and exosomal proteome of plasma, urine and saliva obtained from published literature and in silico databases followed by a comparative analysis using FunRich2.0.2. PPI network of exosomal proteome was analyzed using STRING10.The workflow is as depicted in figure 1.

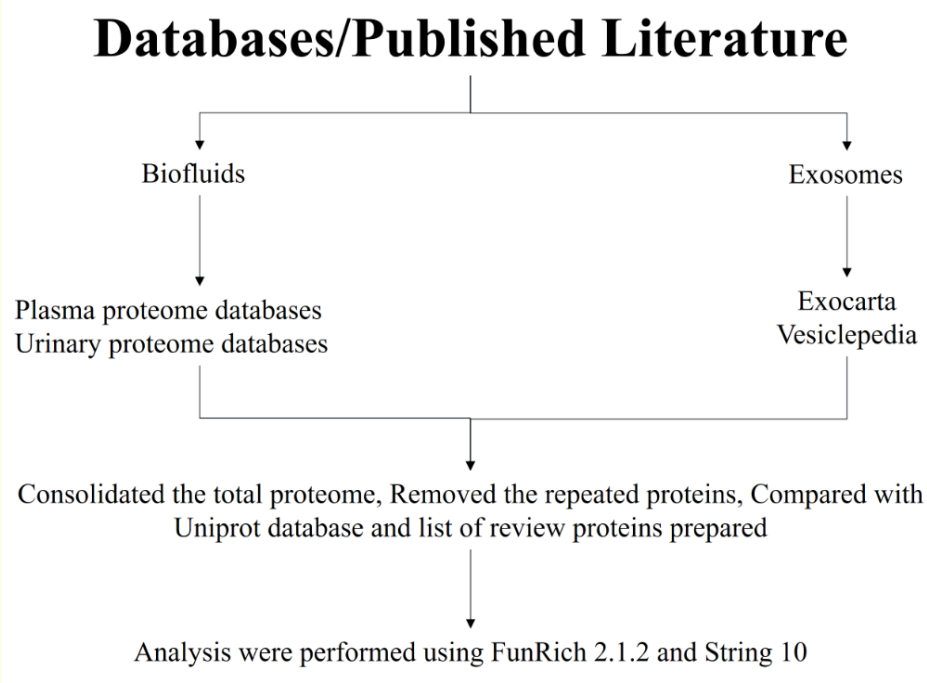


Figure 1

Results

Comparison of exosomal and total proteome of plasma, saliva and urine

Exosomes secreted from cells are known to be carried to their target cells via body fluids [13] and have also been considered as biomarkers of disease which include, cancer and neurodegenerative diseases [11]. To answer our query, we enumerated the total and exosomal proteome of saliva, plasma and urine from published literature [16] and databases (Exocarta, Plasma proteome database and Vesiclepedia). Repetitive and unreviewed proteins were removed and a consolidated proteome was prepared.

We obtained a list of 10,472 plasma proteins,1,194 salivary proteins and 1,758 urinary proteins recorded in healthy donors. Similarly, we consolidated the exosomal proteome of plasma, saliva and urine. We catalogued 291 plasma exosome proteome, 489 salivary exosome proteome and 1,574 urinary exosome proteome. This was followed by a comparative analysis of the total proteins among the three biofluids (Figure 2A). Our analysis showed that only 3.8% of proteins were overlapping among the plasma, saliva and urine. This shows that the proteome of biofluids are very specific. Interestingly, we found 58.29% of salivary proteome to match with plasma proteome agreeing to the earlier report where 27% of similarity between whole saliva proteins and plasma proteins have been reported [17]. This might be due to difference in the resolution of proteomics technology used. In case of urine, 47.15% of urinary proteome matched with the plasma proteome. This might be due to urinary protein variation depending on time of collection and the stability of urinary proteins unlike serum, prone to proteolytic degradation [18].

Further, we analyzed the exosomal proteome isolated from plasma, saliva and urine. On a comparative analysis, we found 2.4% of proteins to be common among the exosomal proteome of plasma, saliva and urine (Figure 2B) while 2.2% of proteins overlapped between salivary exosome and plasma exosome proteome. We also found that 6.09% of urinary exosome proteome is similar to that of plasma exosomes. This signifies that the exosomal proteins are specific since the percentage of overlapping proteins of exosomes from plasma, saliva and urine is comparatively lesser than that of the total proteome of respective biofluids. On comparison, 86.25% of plasma exosomal proteins were found to be contributory of total plasma proteome (Figure 3A). Similarly, 54% of salivary exosome proteome contributed to the total salivary proteome and 43.64% of the urinary exosome proteins contributed to the total urine proteome (Figure 3B and 3C). Our results indicate that the exosomes released from cells transport proteins to biofluids (plasma, serum, saliva) and contribute significantly to the total proteome of the biofluids. Thus, isolation of cell-specific exosomes from biofluids will be the next open challenge for exosome researchers.

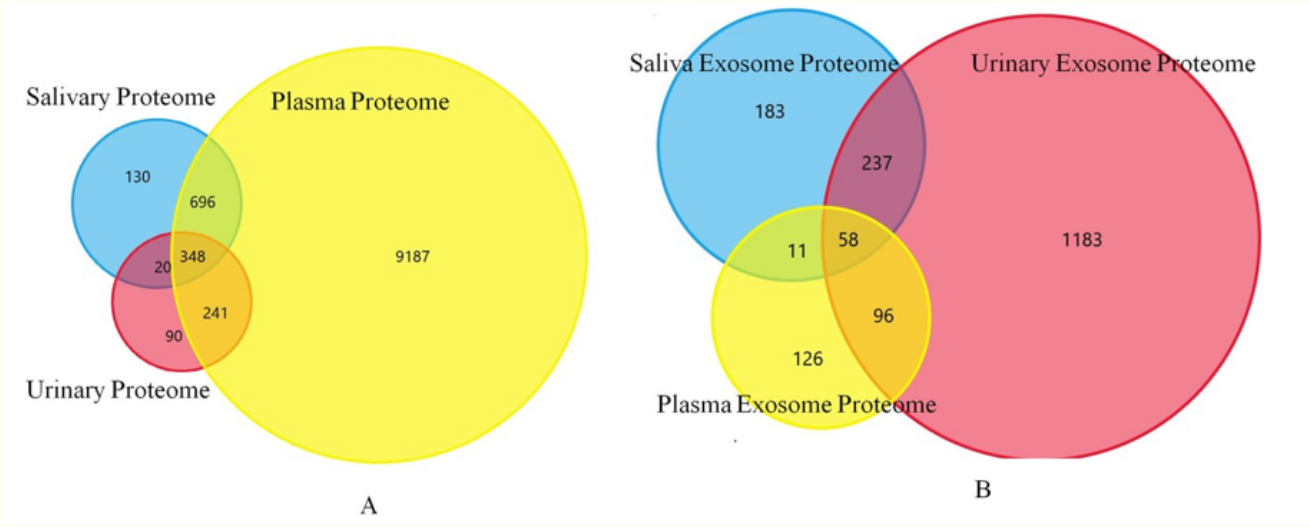


Figure 2: *Comparative analyses of (A) Total proteome of plasma, saliva and urine (B) Exosomal proteome of plasma, saliva and urine.*

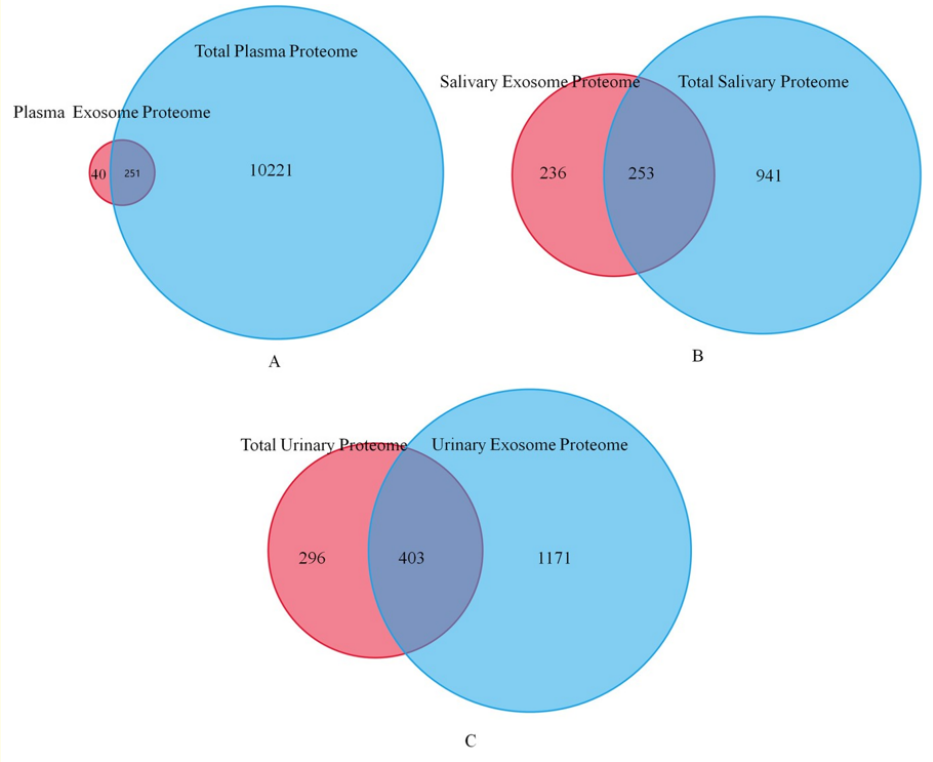
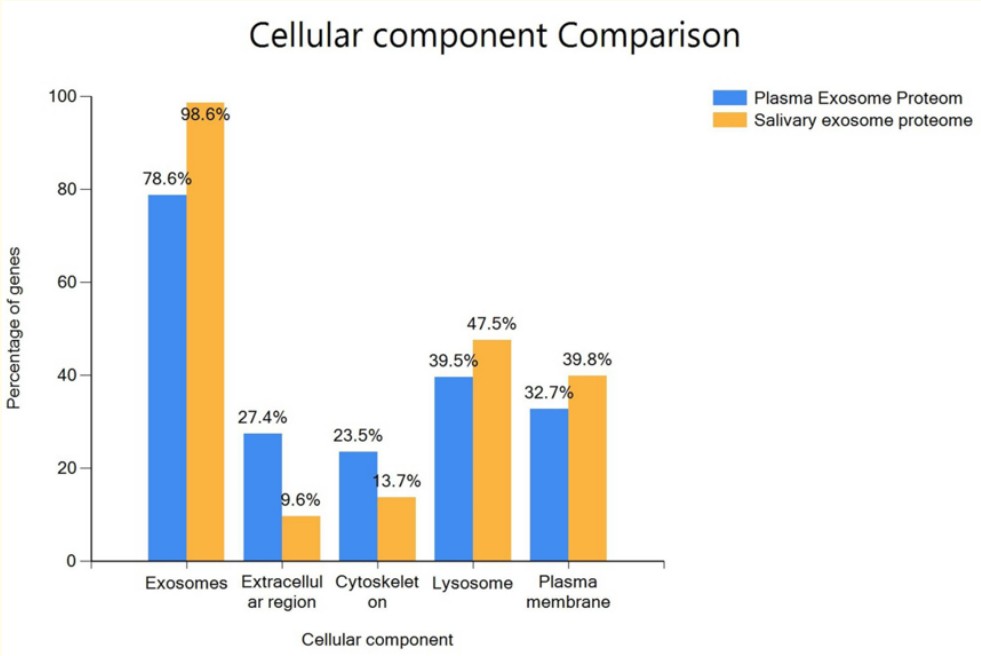


Figure 3: *Comparative analysis of exosomal proteome with their corresponding biofluids (A) Plasma exosomal proteome with the total plasma proteome (B) Salivary exosomal proteome with the total salivary proteome (C) Urinary exosome proteome with the total urinary proteome.*

Protein-protein interaction analysis using STRING 10

The exosomes originate from cells and may exhibit a vesicular protein- protein interaction network similar to other intracellular organelles like, mitochondria, phagosomes. Our data in concordance to the earlier reports indicate that exosomes contain a specific sub-population of proteins rather than randomly selected proteins [19]. The specificity of exosomal proteins depend upon their source of isolation and cell-type specific components. Since the exosomal proteins are specific and the proteins of exosomes have functional role, we further analyzed the exosomal proteome for PPI network using STRING 10 (Figure 4). Thus, the Uniprot id of protein was used as input and the following parameters were set in STRING 10 online tool (http://string-db.org/): High confidence level of (0.700) and only those experimentally-proven interactions were considered. STRING Database showed the co- expression, presence of different interacting partners within plasma exosome, salivary exosome and urinary exosome (Figure 5A-5C). PPI networks clearly indicate that the exosomal proteins are interconnected to form functional nodules agreeing to earlier reports [20]. PPI network of exosomal proteins indicate the presence of several hub proteins with many other exosomal proteins having few interacting partners. Our results also indicated that the physically interacting cellular proteins may have been co-sorted into exosomes. However, further experiments are required for detailed understanding of the molecular mechanism.



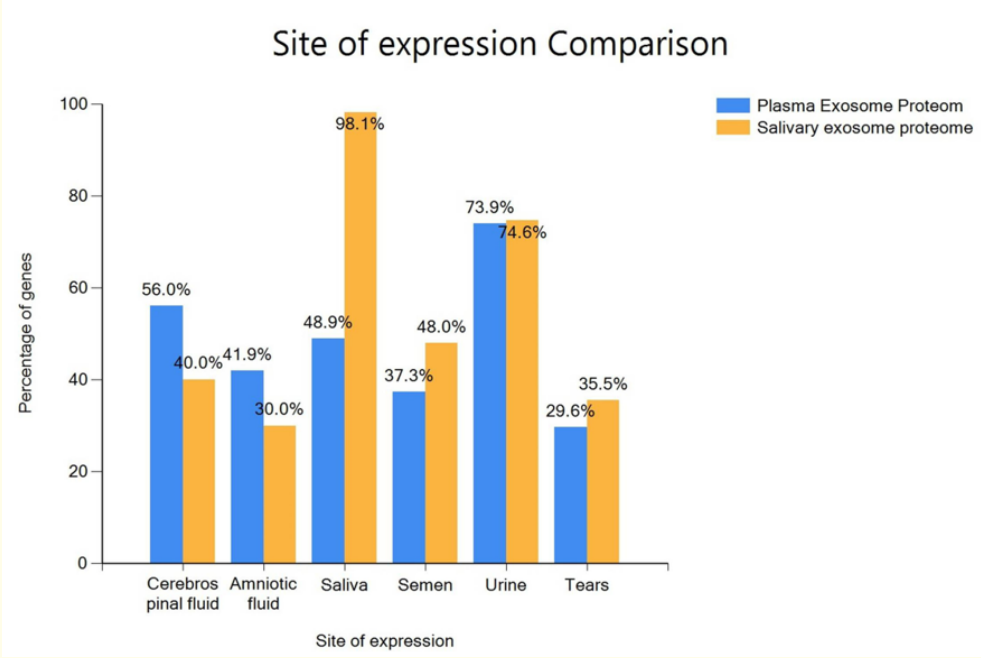


Figure 4: *Functional enrichment analysis of exosomes of saliva and plasma (A) Cellular component (B) Site of expression.*

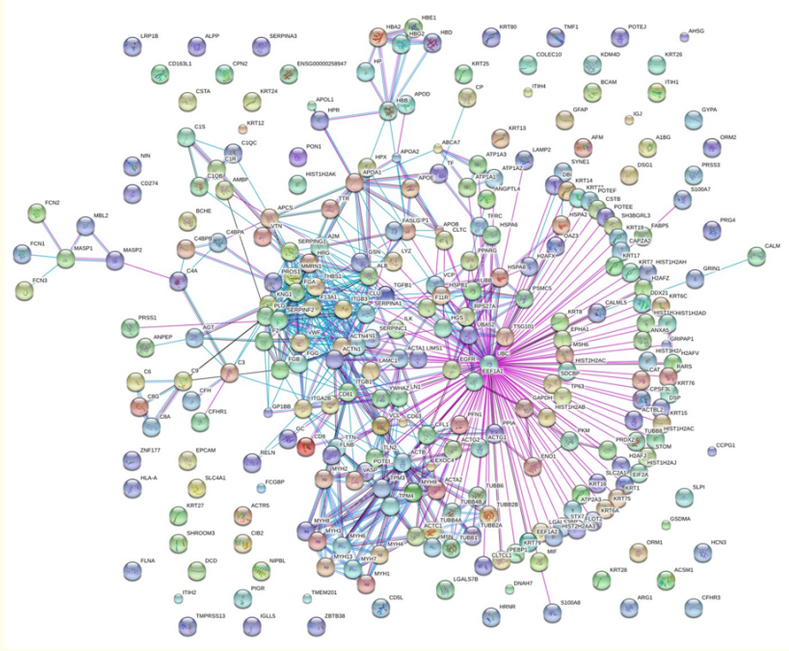


Figure 5A

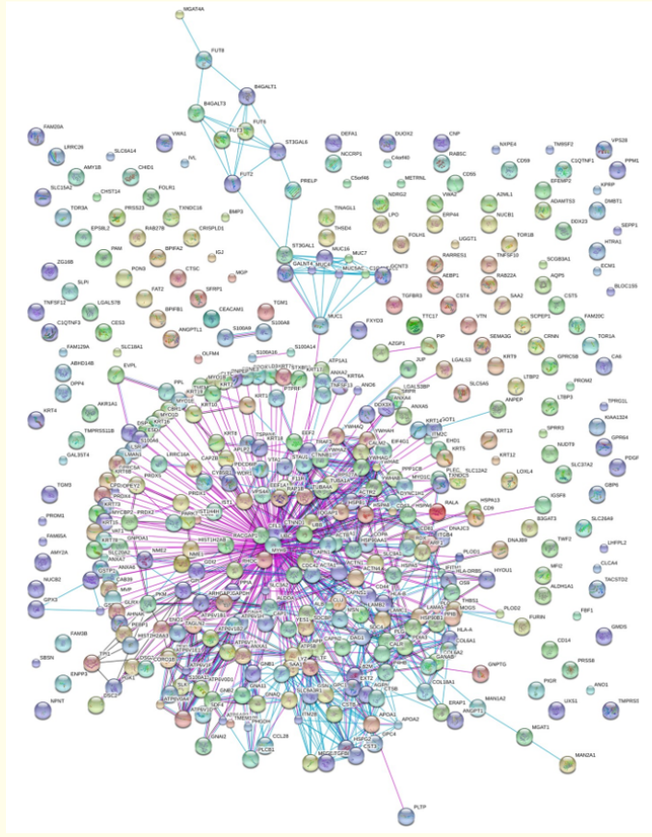


Figure 5B

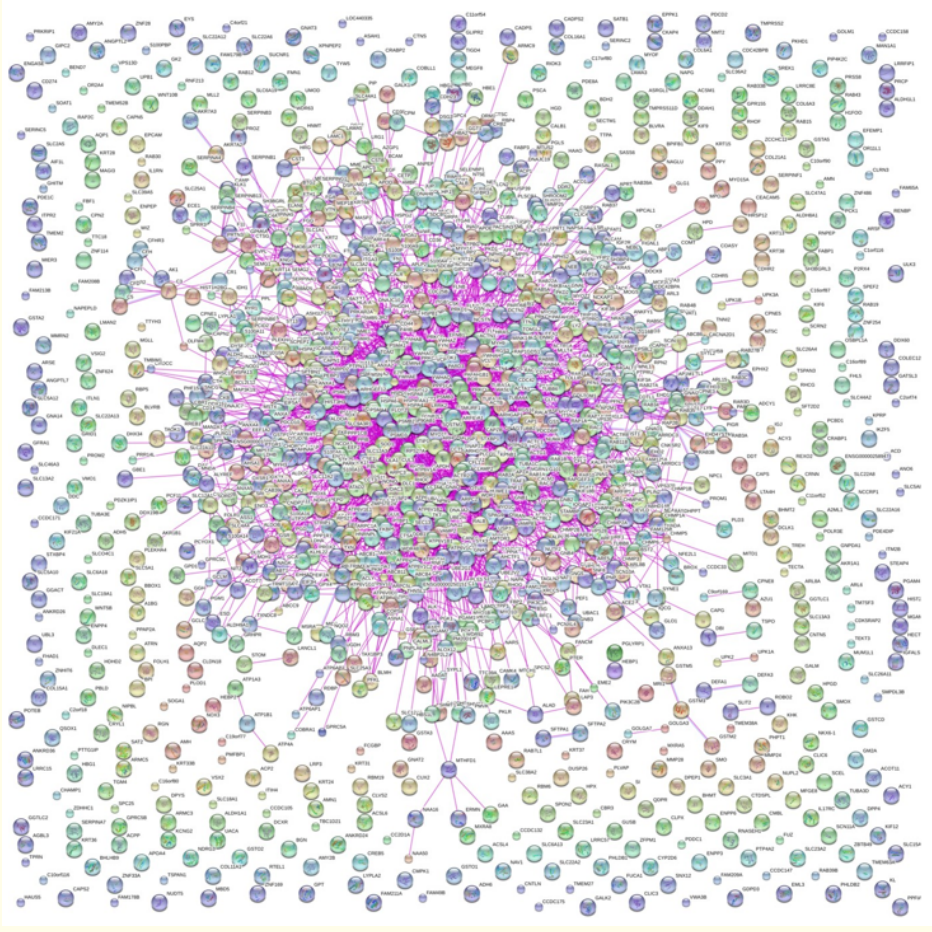


Figure 5C

Figure 5: *Possible interacting partners in (A) Plasma exosomes (B) Salivary exosomes (C) Urinary exosomes.*

Discussion and Conclusion

Cells release different types of extracellular vesicles and exosomes are one of them [21]. As purification technology and proteomic analytical techniques developed further, studies that highlight the number of vesicular proteins present in a single type of extracellular vesicle or in a single extracellular vesicle will be of great importance to understand the exosome biology. In the recent years, exosomes have been considered as key determinants of intracellular communications [13]. Apart from their role in intracellular communication, they are also considered to be indicative of the cell type by which they are released [19]. The presence of tissue-specific markers in exosomes may help in the identification of the exosome specific to a biofluid where different pools of exosome exist. Our data clearly indicate that the proteome of exosome from plasma, saliva and urine are specific and share only 2.4% of proteins in common. Similarly, the total proteome of the biofluids share only 3.8% of proteins in common. As reported earlier [17] and we also found that 54% of salivary proteome found in plasma, interestingly only 2.2% of salivary exosome proteome matches with plasma indicating that the exosomal proteome are much more specific compared to the total biofluid from where it has been isolated. However proteome of salivary exosomes shares around 48.46% of similarity with urinary proteome. Apart from the specific nature of exosomes, other interesting observation was the absence

of highly abundant proteins in the exosomes which masks the detection of non-abundant proteins. Hence the specific nature of exosomes should be considered when used for targeted drug delivery.

Many studies on proteomics of biofluids have been done for the discovery of disease related signature and protein biomarkers or novel protein targets for therapeutic interventions [22]. These studies have identified a list of differentially regulated proteins in diseased state compared to control. These proteins were proposed as biomarkers or targets for therapeutic interventions [23]. The presence of these proteins in exosomes isolated from the respective biofluids has intrigued researchers to use exosomes as an alternative to the corresponding biofluid. For example, CD44 of saliva specimen considered as a biomarker in Head and Neck squamous cell carcinoma [24] is also present in the salivary exosome proteome [25]. Similarly, CD59, a biomarker for oral squamous cell carcinoma [26] is also present in the exosome of salivary proteome. Proteins like PCA-3 and TMPRSS2, known biomarkers of prostate cancer and kidney disorder are also present in the urinary exosomes [11]. Thus, any change in the exosomal proteome is indicative of the pathophysiological condition of cells and the biofluids, making exosomes a valuable tool for study.

Further, PPI network was visualized using STRING 10. As reported earlier by Choi., *et al*. exosomal proteins were interconnected via physical interactions [20]. This also indicates that the exosomal proteins have interacting partners. These interactions might be due to the presence of physically interacting cellular proteins which might have been co-transported into the exosomes. Thus, the proteins present in exosomes are in their active form and exert their potential to interact with each other which provides an insight that exosomes have the potential to be a model to study PPI in biofluids.

Authors Contributions

AC, JN have conceived the idea, AC did the experiment, and both have analyzed and wrote the manuscript.

Acknowledgement

We are thankful to Dr Rama Rajaram and Rebecca Manohar for critically analyzing and correcting the manuscript.

Conflict of Interest

All authors declare no conflict of interest.

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Volume 12 Issue 6 June 2021