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Proteomic Characterization of Cardioprotective Human Acellular Amniotic Fluid

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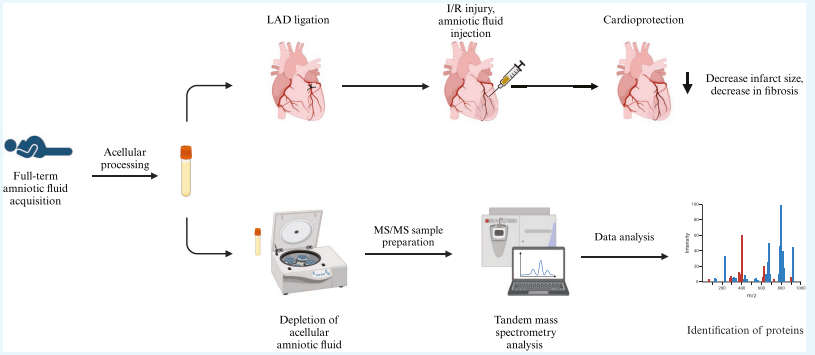

ABSTRACT: Amniotic fluid-derived products are a promising resource for cell therapy and tissue engineering due to their anti-inflammatory, angiogenic, and antifibrotic properties. Human amniotic fluid (hAF) has been used in medical applications such as wound healing, skin disorders, and ophthalmic conditions. Recently, we demonstrated that hAF is an effective treatment for myocardial ischemia-reperfusion injury in adult rats. However, the protein composition of full-term acellular hAF has remained poorly characterized. To uncover the biologically active components underlying hAF's cardioprotective effects, we conducted a global proteomic analysis of hAF collected from six patients at full-term cesarean sections. Previously shown to improve cardiac function in ischemic rats, these samples were analyzed by using tandem mass spectrometry. We identified 657 proteins, including 148 unique to the deep learning platform Inferys. Bioinformatic analysis revealed that these proteins are involved in immunity, inflammatory responses, cell adhesion, and apoptotic signaling pathways. In addition, these proteins were highly modified, with methylation and deamidation being the most abundant modifications. This study represents the first mass-spectrometry-based characterization of full-term, acellular hAF, suggesting that hAF offers a wide array of immune-modulating proteins working together to provide robust cardioprotection and a valuable treatment for ischemia-reperfusion injury.

INTRODUCTION

Amniotic-derived products, including mesenchymal stem cells, epithelial stem cells, epithelial cells, and acellular scaffolds, have long been considered a promising resource for cell therapy and tissue engineering.[1−3] In particular, amniotic fluid (AF) possesses robust and diverse biological properties, including immune-modulatory, anti-inflammatory, angiogenesis, and antiscarring activities.[4,5] Unlike embryonic stem cells or their derivatives, which have ethical concerns, amniotic fluid does not carry these same concerns and is usually discarded after childbirth. In addition, to avoid several ethical and biological challenges or potential adverse effects associated with allograft use of multipotent stem cells, amniotic fluid can be further processed to remove intact cells and cellular debris to generate acellular hAF.[6]

We developed and implemented a proprietary method to generate acellular fractions of human amniotic fluid (hAF). When filtered and sterile processed, hAF is a nonantigenic solution, rich in hundreds of proteins involved in immunomodulatory, extracellular matrix, antimicrobial, fibrosis, and cellular growth pathways.[7,8] Amniotic fluid has been safely applied to patients with burn wounds, epithelial eye injuries, and COVID-19.[9−14] More recently, we reported that hAF dramatically reduced myocardial ischemia-reperfusion injury in rodents.[15] While we presented several hypothesis-generating data in our previous publication, the mechanism of the salutary effect of hAF remains unknown.

Several studies[16−24] have previously characterized the hAF proteome unbiasedly using tandem mass spectrometry (Table 1). However, most of these studies were conducted on second-trimester samples (when amniocentesis is commonly per-

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Table 1. Published Studies of Mass Spectroscopy Based Analysis of Human Amniotic Fluid

manuscript	acellular	depletion	weeks
1. Nilsson et al.[16]	no	yes	15
2. Tsangaris et al.[17]	no	no	16−18
3. Park et al.[23]	no	no	39
4. Tsangaris et al.[18]	no	no	16−18
5. Michaels et al.[24]	no	yes	11−13, 16−18, >18
6. Cho et al.[19]	no	yes	16−18
7. Cho et al.[20]	no	yes	16−20
8. Liu et al.[21]	no	yes	16−20
9. Shorey-Kendrick et al.[22]	no	yes	20, 26, 33
10. this manuscript	yes	yes	39−40

formed),[7] and all of these examined amniotic fluid that had not been subjected to fractionation to remove intact cells. Only one additional study, which utilized an aptamer-based array, has examined acellular hAF.[25] Considering the extensive variation in amniotic fluid constituents throughout gestation and potential confounding cellular contribution, we sought to characterize the proteome of full-term acellular hAF to understand its role in complex biological pathways. In addition, we chose to characterize samples with established biological activity (the same samples previously utilized in rats and shown to reduce myocardial ischemia-reperfusion injury) to begin to decipher the molecular mechanisms driving their ability to protect the heart.

RESULTS

In this study, we performed a global proteomic analysis of six human acellular amniotic fluid (hAF) samples (Figure 1A) previously shown to be cardioprotective.[7] Specifically, in a rat model, these samples were shown to reduce infarct size and pathological remodeling after ischemic reperfusion injury. These amniotic fluid samples were collected at 39−40 weeks from patients with full-term elective cesarean section surgeries, as previously published.[7] The median gestational age was 39 weeks, with additional demographics included in Figure 1. After the acquisition, the fluid was processed from volunteer donors via aseptic techniques followed by proprietary filtration technology to obtain sterile hAF. Sterile acellular hAF was either depleted using the top 12 depletion resin columns from Pierce or not depleted, as depicted in Figure 1B. After depletion (or no depletion), samples were processed utilizing filter-aided sample preparation and enzymatic digestion for global mass spectrometry analysis. Peptides were loaded onto a C18 analytical column, and ionized peptides were analyzed on an Orbitrap LTQ Velos Pro and fragmented by collision-induced dissociation (CID) or higher energy collision-induced dissociation (HCD). Data sets were then analyzed in Proteome Discoverer version 2.5 or Peaks version 7.5 to identify post-translational modifications in hAF, as shown in Figure 1B.

Depletion of highly abundant proteins present in biological fluids (including albumin and IgG) is a common strategy to increase the analytical depth and improve the detection of low abundant proteins. To confirm the efficiency of sample depletion while maintaining total protein content, we graphed the abundance of depleted and nondepleted amniotic fluid analyzed by mass spectrometry, demonstrating that similar amounts of total protein were analyzed from each sample (Figure 2A). The circles represented in the graph are highly abundant proteins (albumin, transferrin, and IgG′s) commonly

A

Donor	Donor Age, (yr)	Gestational Age	Ethnicity	Reason for Elective CS
#1	33	39 wk, 0 day	Caucasian	Prior CS
#2	32	39 wk, 0 day	Caucasian	Prior CS
#3	20	40 wk, 5 day	Caucasian	Macrosomia
#4	37	39 wk, 3 day	Asian	Prior CS
#5	37	39 wk, 2 day	Asian (F) Caucasian	Prior CS
#6	39	39 wk, 0 day	Black (F) Caucasian	Prior CS

B

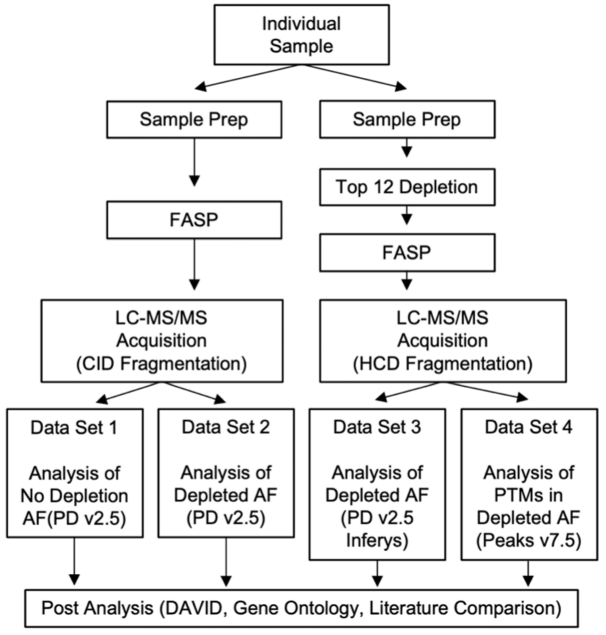

Figure 1. Patient demographics and study design schematic. (A) Allogenic human amniotic fluid (hAF) was collected and processed as previously described from volunteer donors who met medical and social history criteria as established by the Federal Food and Drug Administration (FDA), the United States Public Health Service (USPHS), and the American Association of Tissue Banks (AATB). Collection of hAF into sterile Medi-Vac containers occurred during a scheduled cesarean section, as approved by the Institutional Review Board at the University of Utah, and was transported to the Cell Therapy and Regenerative Medicine (CTRM) facility. The hAF was transferred via aseptic techniques and processed using a proprietary filtration technology to obtain sterile acellular hAF. (B) Acellular amniotic fluid was analyzed through two major pipelines as depicted, with the samples being either nondepleted or depleted of the most abundant proteins. Samples were then digested and analyzed by using CID or HCD fragmentation. In our mass spectrometry analysis of hAF, we integrated multiple analytical variations, including different fragmentation methods and analysis software, to achieve the highest coverage of proteins and post-translational modifications.

found in human serum and plasma samples. After the depletion, protein abundances fell within the rectangle representing the second and third quartiles of the data set. We confirmed the reproducibility of biological and technical replicates using principal component (PC) analysis in Figure 2B. All proteins identified by CID in depleted and nondepleted samples were displayed in a heatmap, depicting alterations in protein abundance profiles (Figure 2C). In addition, Figure 2D highlights the 44 proteins depleted from the amniotic fluid samples, including highly abundant proteins such as albumin, transferrin, fibrinogen, and IgGs. Our depletion of the most

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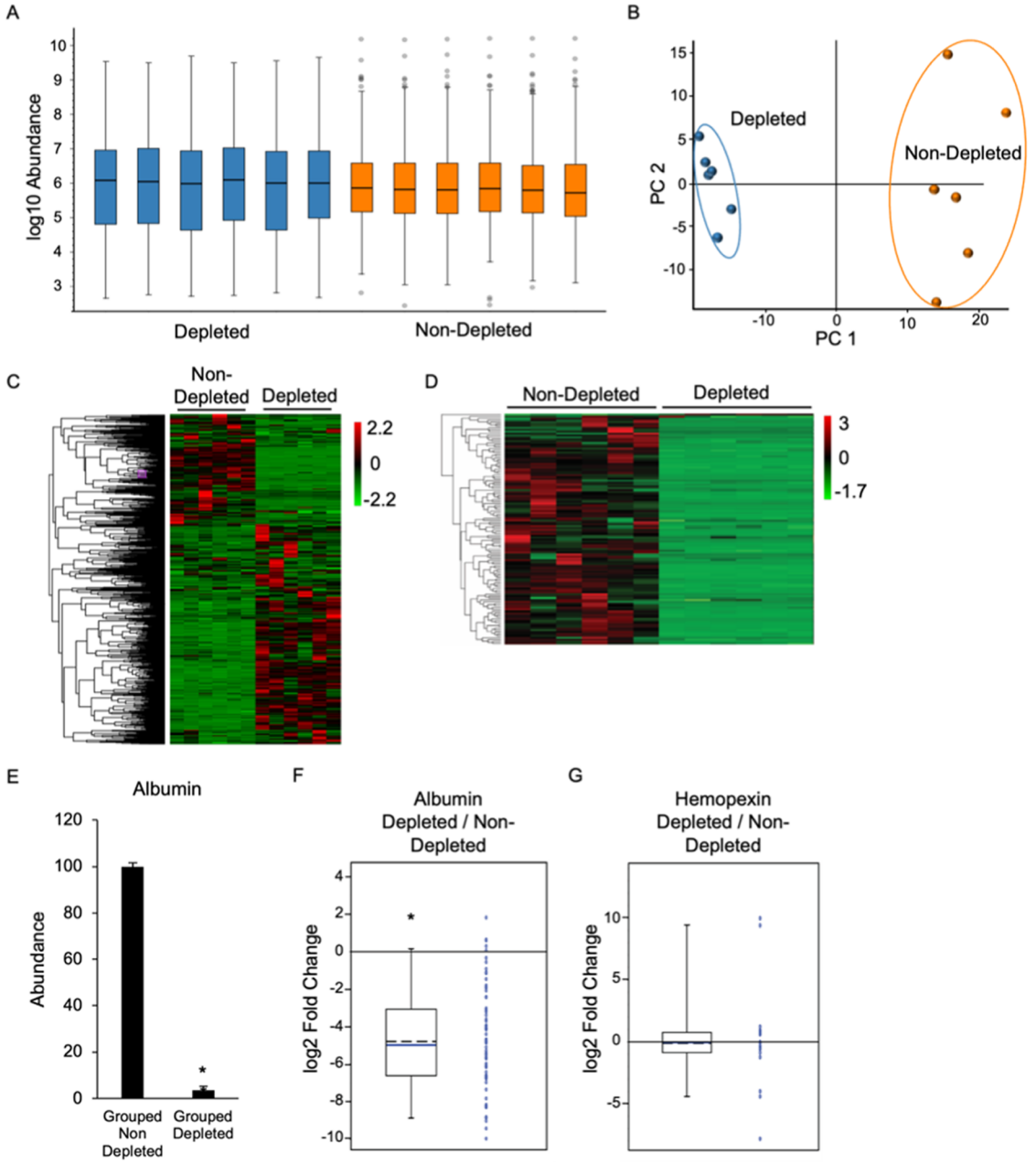

Figure 2. Efficacy of protein depletion in human acellular amniotic fluid. Depleting the most abundant proteins from amniotic fluid, such as albumin, transferrin, fibrinogen, and IgGs, increased coverage and overall sensitivity of low abundant proteins. (A) Sample abundance (log10) of nondepleted (orange) and depleted (blue) amniotic fluid confirms the efficiency of sample depletion while maintaining total protein content. (B) Two-dimensional principal component (PC) analysis showed a distinct separation of nondepleted (orange) and depleted (blue) amniotic fluid samples, confirming the reproducibility of biological and technical replicates. (C) Heatmap of all proteins identified by CID after depletion, and (D) the 44 depleted proteins. (E) Quantitation of albumin shows a 95% reduction in abundance across all depleted samples. (F) A comparison of albumin abundance, showing depletion, versus (G) hemopexin abundance, showing no change, confirms the efficacy and specificity of the depletion strategy (blue dots represent peptides quantified). The asterisk indicates p-values less than 0.05.

abundant proteins led to increased coverage and sensitivity, with a 95% reduction in albumin abundance observed across all depleted samples (Figure 2E/F). Additionally, we confirmed the specificity of the depletion kit using a representative protein (Figure 2G), where hemopexin abundance remains unchanged post depletion, indicating only targeted removal of select proteins without nonspecific effects.

In our mass spectrometry analysis of hAF, we integrated multiple analytical variations, including different fragmentation methods and analysis software, to achieve the highest coverage of proteins and PTMs. Overall, in this study, we identified 657 proteins, with 149 uniquely identified using collision-induced dissociation (CID), 96 using higher energy collision dissociation (HCD), and 412 proteins identified using both techniques (Figure 3A and Supporting Table 1). Within this data set, using the HCD data, we applied both a conventional workflow in proteome discoverer utilizing Sequest HT and the deep learning rescoring algorithm Inferys 1.0 (only compatible with HCD data), which increased our identification from 360 proteins to 504 proteins within this data set (Figure 3B, Supporting Tables 2

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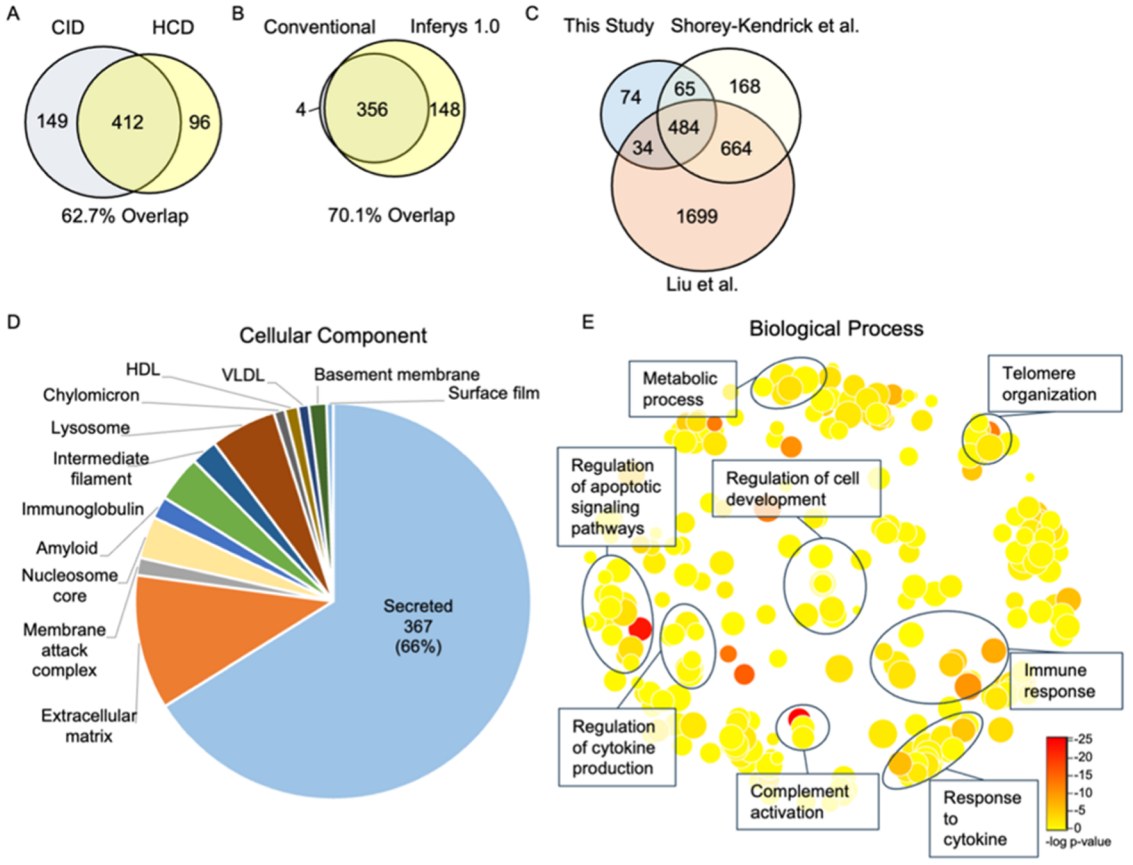

Figure 3. Proteomic analysis of a full-term human amniotic fluid. (A) Venn diagram of all 657 identified proteins using CID or HCD fragmentation. (B) Venn diagram comparison of protein identification in a conventional HCD data analysis pipeline and using the deep learning platform Inferys 1.0 in Proteome Discoverer v2.5. (C) Data set comparison of proteins identified in this study with those identified by Liu et al. and Shorey-Kendrick et al., which showed a 78.8 and 83.6% overlap, respectively, despite differences in gestation time and sample processing. (D) Gene Ontology (GO) analysis was performed on the 657 proteins identified in the amniotic fluid that provided cardiac protection. Cellular component analysis showed that a majority of proteins are secreted. (E) Biological Process terms of the 657 proteins identified were processed in REVIGO to remove redundant gene ontology terms and to visualize the data. Bubble color in the scatterplot indicates the -log p-value associated with the GO term; size indicates the frequency of the GO term in the EBI GOA database (General GO terms are larger).

and 3).[26] More specifically, the Inferys rescoring platform increased the identification of PSMs by 30%, 37% in the peptide group, and 44% in the protein group (Figure S1A). When comparing proteins identified on a per-sample basis, we showed 96% similarity in the proteins identified across all nondepleted samples and 95.9% across all depleted samples, highlighting the consistency of hAF proteins in the third trimester (Figure S1B).

We then compared our data with the two most recent papers that analyzed human AF (albeit at different gestational times) utilizing liquid chromatography with tandem mass spectrometry (LC-MS/MS) (namely, the studies by Liu et al. and Shorey-Kendrick et al. listed in Table 1 and Supporting Table 5). We did not perform a comparative analysis of the additional studies listed in Table 1 due to the substantial differences in instrumentation (Maldi-TOF), sample preparation (2-D gel electrophoresis), nuances in data acquisition, stringency for protein identification, and the use of nonhuman databases, detailed in Supporting Table 5. When comparing our data to both Liu et al. and Shorey-Kendrick et al., both of which performed a global proteomic analysis of amniotic fluid collected during amniocentesis (16−33 weeks of gestation), we observed 78.8 and 83.6% overlap in proteins (Figure 3C) despite the differences in gestation and fluid processing. In addition, we also cross-validated the rescored proteins from Inferys 1.0 (148 proteins). We observed an 80.4% overlap in proteins identified by Inferys with Shorey-Kendrick et al., highlighting the reproducibility of the Inferys algorithm (Figure S1C). We then performed Gene Ontology analysis in DAVID on the 657 total proteins identified in this study. Cellular component analysis classified the majority of proteins as secreted proteins (66%), followed by extracellular matrix proteins (11%) and nucleosome core proteins (3%) (Figure 3D). Biological process analysis terms with a p-value (<0.05) were then processed in REVIGO to reduce redundancy and visualize the data. REVIGO analysis (Figure 3E) identified immune system processes (represented by "immune response", "response to cytokine", "complement activation," and "regulation of cytokine production") and regulation of apoptotic signaling pathways as two of the pathways most represented in hAF.

Next, we investigated the post-translational modification (PTM) landscape of the proteins in hAF. Using these methods, 292 proteins were shown to contain at least one PTM, with the most common modifications identified being methylation (163 proteins), deamidated (102 proteins), demethylation (86 proteins), phosphorylation (71 proteins), acetylation (56 proteins), and trimethylation (26 proteins), Figure 4A. Interestingly, of the 292 proteins identified with a PTM, 275 (43.5%) were methylated with a single methyl group or

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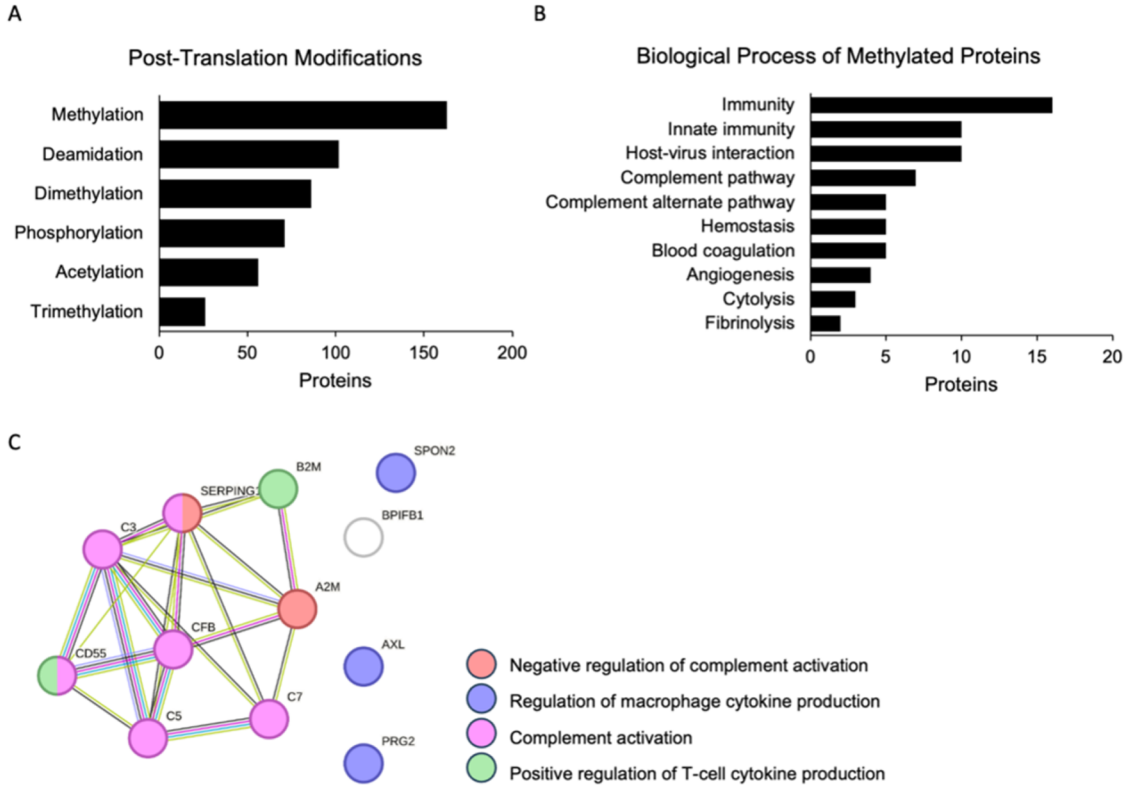

Figure 4. Post-translational modifications identified on proteins present in a full-term human acellular amniotic fluid. (A) Of the 657 proteins identified, 292 were found to contain at least one post-translational modification, including methylation (163), deamidation (102), demethylation (86), phosphorylation (71), acetylation (56) and trimethylation (26). (B) Gene ontology analysis of the methylated proteins was performed, and a strong involvement of "immunity" or immune modulation was observed. (C) Proteins annotated with "immunity" were subjected to network mapping using STRING, further highlighting their interconnectivity and specific involvement in immune modulation.

combination of monomethylation, dimethylation, and trimethylation. To further interrogate the biological processes that these proteins are involved in, we subjected methylated proteins to gene ontology analysis. We identified immunity, innate immunity, complement pathways, and angiogenesis as some of the most enriched processes. In addition, bioinformatic analysis using the STRING database integrated the specific methylated proteins involved in "immunity" and mapped their known interactions and particular roles in immunity (Figure 4C). This analysis supports the involvement of these processes, complement activation, regulation of macrophage cytokine production, and positive regulation of T cell cytokine production as one mechanism by which hAF can influence cardiac pathology and physiology.

DISCUSSION

Our global proteomic analysis identified 657 proteins in full-term sterile acellular hAF, including 292 proteins containing at least one post-translational modification, likely critical to its cardioprotective mechanisms. In our study, we applied a highly abundant depletion kit to remove proteins including albumin, transferrin, fibrinogen, and immunoglobulins, enhancing protein coverage and PTM identification. Notably, this process led to a 95% reduction in albumin abundance across all depleted samples, facilitating the identification of low-abundance proteins that may contribute to hAF's therapeutic effects.

Gene ontology analysis revealed that most identified proteins (66%) are secreted, with significant representation in the extracellular matrix and lysosome components. The presence of proteins involved in immune system processes and cell death regulation pathways was particularly notable, suggesting a multifaceted mechanism of action for hAF. Furthermore, identifying 292 proteins with PTMs, including methylation, demethylation, and phosphorylation, highlights the intricate molecular modifications contributing to the functionality of hAF.

Detailed analysis of methylated proteins indicated a significant influence on immune processes such as complement activation and cytokine production regulation. This is consistent with our in vivo observations of decreased inflammatory cytokine expression and immune-modulatory markers. Despite the presence of ongoing myocyte injury, the significant reduction in the infarct size and fibrosis implies a robust protective response mediated by hAF.

When comparing our data with two other studies by Liu and Shorey-Kendricks et al., we observed significant overlap with AF collected at different gestational times, although there also appear to be unique temporal changes to the proteins in amniotic fluid over the course of gestation. We did not compare our data from human amniotic fluid to any other species, including rats or mice, as we would like to translate its application to clinical studies. Currently, no studies have been published that utilize mouse or rat amniotic fluid for cardiac ischemic injury. While this could be an interesting comparison, it would be limited in its clinical relevance and translation for use in humans.

The clinical relevance of hAF is underscored by its potential applications in myocardial infarction, cardiac surgery, and organ transplantation to attenuate ischemic injury. Unlike other

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amniotic products containing cellular components, this hAF is acellular, minimizing immunogenicity and tumorigenicity risks. Our preclinical studies and clinical trials have demonstrated the safety and efficacy of intravenous hAF, with consistent benefits observed across multiple donors, suggesting scalability for therapeutic use.

While this study advances our understanding of the protein constituents in full-term human amniotic fluid and highlights changes in this proteome during the third trimester, utilizing mass spectrometry may not identify very low abundant proteins/peptides. In addition, the exact bioactive component(s) have not been identified on an individual basis, although most hypotheses suggest that its bioactivity is the result of a combination of molecules. Therefore, future studies will be necessary to further interrogate the foundation of their bioactivity in different tissues.

CONCLUSIONS

While it remains challenging to delineate a singular mechanistic pathway, our findings support that full-term hAF operates as a natural repository of bioactive elements, collectively contributing to its cardioprotective effects. This study underscores the potential of hAF as a versatile therapeutic agent that is widely accessible for acute cardiac injury, leveraging the evolutionary complexity of its molecular composition.

METHODS

Amniotic Fluid Acquisition. Allogenic human amniotic fluid (hAF) was collected and processed as previously described[7] from volunteer donors who met medical and social history criteria for cell and tissue donor acceptability, as established by the Federal Food and Drug Administration (FDA), 21 CFR 1271, the United States Public Health Service (USPHS), and the American Association of Tissue Banks (AATB). Collection of hAF into sterile Medi-Vac containers occurred during scheduled cesarean sections as approved by the Institutional Review Board at the University of Utah. It was transported to the University Cell Therapy and Regenerative Medicine (CTRM) facility. An aliquot of hAF was removed and designated for preprocessing and microbiological testing for aerobic, anaerobic, and fungal microorganisms. Then, the hAF was transferred via an aseptic technique and processed using proprietary filtration technology to obtain sterile acellular hAF.

Depletion Kit. Before tandem mass spectrometry analysis, a portion of the sterile acellular human amniotic fluid was depleted using Pierce top 12 abundant protein depletion spin columns (85615). The depletion spin column was equilibrated to room temperature, and hAF was added directly to the resin slurry column, which was capped and then inverted several times. The sample was then incubated at room temperature for 60 min with gentle end-overend mixing. After incubation, the column was placed into a collection tube and was centrifuged at 1000x g for 2 min. The filtrate that contained the sample (minus the depleted proteins) was stored in 10 mM PBS, 0.15 NaCl, and 0.02% sodium azide, pH 7.4.

Sample Preparation. Samples were digested as previously published.[27,28] Briefly, depleted and nondepleted amniotic fluid (10 μg) from each of the six samples was loaded into separate Vivacon 500 filter units, concentrated at 13,000*g*, and then washed 3 times with 100 μL of urea buffer (8 M Urea, 0.1 M Tris/HCl pH 8.5). The concentrate was mixed with 100 μL of 50 mM iodoacetamide in urea buffer and incubated at room temperature in the dark for 20 min, followed by centrifugation for 15 min at 13,000*g*. The concentrate was washed twice with 100 μL of urea buffer and two washes with 100 μL of 50 mM ammonium bicarbonate. Ten μg of protein from each sample was mixed with trypsin (1:40) in the filter and incubated overnight at 37 °C. The peptides were then eluted with 50 mM ammonium bicarbonate and acidified with 1% formic acid.

Mass Spectrometry Analysis. Tryptic peptides were analyzed as previously published[27−30] by nanoflow LC-MS/MS on a Thermo Orbitrap Velos Pro interfaced with a Thermo EASY-nLC 1000 equipped with a reverse-phase column (75 μm inner diameter, 360 μm OD, 15 cm, Reprosil-Pur 120 C18 AQUA 3 μm particle size; ESI Solutions) and a flow rate of 400 nL/min. For peptide separation, a multistep gradient was utilized from 98% Buffer A (0.1% formic acid, 5% DMSO) and 2% Buffer B (0.1% formic acid, 5% DMSO in acetonitrile) to 10% Buffer A and 90% Buffer B over 90 min. The spectra were acquired using the Nth order double-play, data-dependent acquisition mode for the top 20 most abundant ions in the parent spectra for fragmentation. MS1 scans were acquired in the Orbitrap mass analyzer at a resolution of 30,000. MS1 ions were fragmented by either collision-induced dissociation or higher energy collision-induced dissociation with an activation time of 30 ms and a normalized collision energy of 30. Dynamic Exclusion was enabled to avoid multiple fragmentations of the parent ions.

Data Analysis. The raw files generated were searched against the UniProt human (v2023−11−08) database using Proteome Discoverer v2.5 interfaced with Sequest HT and the Inferys 1.0 search engine.[26] Parameters for Sequest HT were as follows: enzyme trypsin, max missed cleavage site was set to two, precursor mass tolerance was set to 10.0 ppm, and fragment mass tolerance was set to 0.6 Da. The peptides were searched for the static modification of carbamidomethylation on cysteine, dynamic modifications of acetyl (N-terminus), and variable modifications of oxidation on methionine. The false discovery rate for both proteins and peptides was set to 0.01. Proteins were quantified based on unique and razor peptides. Precursor abundance was based on intensity, and normalization was based on the total peptide amount. Protein abundance was calculated by summing sample abundances of the connected peptide groups, and the protein ratio calculation was performed using a pairwise ratio base. Missing intensities were imputated with random values sampled from the lower 5% of detected values. P-values were calculated using a student's t test based on background populations of proteins. Proteins identified were further filtered using the following criteria: the number of peptides is greater than or equal to two, protein FDR confidence is high, and the contaminant is false.

For the analysis of post-translational modifications, raw files were searched against the UniProt human (v2023−11−08) database using Peaks v7.5 utilizing the PEAKS search engine. Parameters for PEAKS were as follows: enzyme trypsin, max missed cleavage site was set to three, max variable ptm per peptide set to 3, precursor mass error tolerance was set to 15.0 ppm, and fragment mass tolerance was set to 0.02 Da. The peptides were searched for the static modification of carbamidomethylation on cysteine, dynamic modifications of Acetyl (N-terminus), and all the variable modifications (478 ptms) built into the PEAKs v7.5 PTM workflow. The false discovery rate for proteins and peptides was 0.01, and De novo ALC was >80%. Proteins identified were further filtered using the following criteria: $-10 \log P$ is equal to or greater than 20,

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and the number of unique peptides is greater than or equal to two.

Gene ontology (GO) analysis of identified proteins used the Database for Annotation, Visualization, and Integrated Discovery (DAVID).[31] When we searched for enrichment of GO terms, a right-sided hypergeometric test was utilized with a Benjamini-Hochberg correction of the p-value. The threshold of the p-value for terms was ≤0.05. Enriched Gene Ontology Terms were then processed in REVIGO,[32] with the following input parameters: resulting list medium (0.7), values associated with GO terms represent p-value, obsolete terms removed, species set to *Homo sapiens* (9606), and semantic similarity set to default. The bubble color in the scatterplot indicates the −log *p*-value associated with the GO term; size indicates the frequency of the GO term in the European Bioinformatics Institute Gene Ontology Annotation database (General GO terms are larger). Bioinformatics analysis of post-translational modifications was performed using the STRING database.[33]

Experimental Design and Statistical Rationale. This study aimed to characterize the composition of full-term human acellular amniotic fluid that provided cardioprotection from myocardial ischemia-reperfusion injury. In this study, we performed global proteomics on six human-derived acellular amniotic fluid samples. The six samples were either depleted or nondepleted and ran on an Orbitrap Velos Pro mass spectrometer (Thermo). Each depleted biological sample was run in duplicates, and each nondepleted sample was run in triplicates on the mass spectrometer. The number of samples and replicates was based on practical considerations, including human sample acquisition and available instrument time. Design and data were formulated and presented according to the previously published suggested guidelines for this model system.[15]

■ ASSOCIATED CONTENT

Data Availability Statement

The MS proteomics data included have been deposited to the ProteomeXchange Consortium[34] via the PRIDE[35] partner repository with the data set identifier PXD053775.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c09451.

Inferys rescoring boosts peptide and protein identification in acellular amniotic fluid (PDF)

Literature of mass spectrometry-based analysis of amniotic fluid; identified proteins In hAF (nondepleted and depleted); identified proteins In hAF Data (HCD conventional approach); and identified proteins In hAF Data (HCD Inferys 1.0 Rescoring) (XLSX)

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Author Contributions

R.B. contributed to data curation; R.B. and S.F. contributed to formal analysis; J.P., S.F., and C.H.S. contributed to funding acquisition; R.B., G.M., H.J., I.N., J.P., S.F., and C.H.S. contributed to investigation; R.B. and S.F. contributed to methodology; J.P. contributed to project administration; S.F., J.P., and C.H.S. contributed to resources; R.B. contributed to software; R.B., S.F., G.M, J.P., and C.H.S. contributed to conceptualization; S.F., J.P., and C.H.S. contributed to supervision; R.B., S.F., J.P., and C.H.S. contributed to validation; R.B. contributed to visualization; R.B., S.F., and C.H.S. contributed to writing—original draft; R.B., G.M., H.J., J.P., S.F., and C.H.S. contributed to writing—review and editing. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare the following competing financial interest(s): The authors declare the following financial interests/personal relationships that may be considered potential competing interests: Several CTRM members are patent holders responsible for developing and manufacturing the acellular amniotic fluid used for these experiments.

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support in collecting and manufacturing hAF. The table of contents image was created with biorender.com.

■ ABBREVIATIONS

hAF, human amniotic fluid; AF, amniotic fluid; LAD, left anterior descending artery; CID, collision-induced dissociation; HCD, higher energy collision-induced dissociation; LC-MS/MS, tandem mass spectrometry; PSM, peptide spectrum match

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