

Age-dependent changes in protein interaction partners of Protein C

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Abstract

Background

Protein C is a critical regulator of haemostasis, exerting anticoagulant, anti-inflammatory and cytoprotective effects through tightly coordinated interactions within the coagulation system. Protein C activity is dependent not only on circulating concentration, but also on its structure and functional interactions with other proteins. The plasma concentration, structure and functional activity of Protein C is known to change with age, but changes in protein-protein interactions (PPIs) of Protein C with age have not been investigated.

Methods

Co-immunoprecipitation (coIP) was performed on plasma samples pooled in age groups spanning from neonates to adults using an anti-Protein C antibody. Eluates were subjected to LC-MS/MS, enriched and depleted proteins identified, and multivariate analysis, pair-wise comparisons, and functional enrichment analysis was performed.

Results

The Protein C co-precipitating proteome was found to change across development, with the most pronounced differences seen in the youngest cohorts. Protein C appears to associate with age-specific haemostatic, platelet, complement, and immune-related protein networks, suggesting that the surrounding protein context of Protein C differs across age. However, pathway analysis identified significant cross-over of pathways in which Protein C and interactors are involved in across age ranges, despite different proteins being identified as participating in these pathways.

Conclusions

The findings of this study highlight global differences in the haemostatic system across normal development, and the complex and shifting role of the plasma proteome with age. Data are available via ProteomeXchange with identifier PXD077907.

Background

Activated Protein C (APC) is a vitamin K-dependent serine protease which plays a vital role in the regulation of blood coagulation. Protein C is activated by thrombin cleavage, releasing a dodecapeptide from the C-terminal domain of the heavy chain (1). APC exerts its anticoagulant role by proteolytic inactivation of the blood coagulation factors Va and VIIIa (2, 3). APC also exhibits various cytoprotective properties, including anti-apoptotic and anti-inflammatory effects, and endothelial and epithelial barrier stabilisation (4). The zymogen Protein C is synthesized by liver parenchymal cells as a single chain polypeptide, which is subsequently cleaved and circulates in plasma in the form of a heavy chain (41 kDa) linked by a disulfide bond to a light chain (21 kDa) (5, 6). Different glycoforms of both the heavy and light chain exist, with slightly different molecular weights (7). The plasma concentration of Protein C in normal healthy adults is ~ 4 µg/mL with a half-life of about 15 hours (8).

Developmental haemostasis describes the age-specific differences in levels and functional activities of most proteins associated with the coagulation cascade (9, 10), and is well-established alongside broader age specific changes in the plasma proteome (11). More specifically, age-related differences in Protein C antigenic and functional levels have been reported, as have structural differences. Protein C levels in healthy neonates have been shown to be approximately 30% that of adults, with levels rising steadily throughout childhood to adult levels (9), while functional levels were found to be around 40% of adult levels in neonates, characterised by a steep rise to around 70–80% of adult levels within the first year of age (10, 12). Exact antigenic and functional levels determined vary with measurement techniques. Further, the presence and relative proportion of different glycoforms, and different antigen to activity levels, of Protein C in newborns and children compared to adults have been reported, as has alternative proteolytic processing (7, 13–15). However, while quantitative differences in protein C levels across the age spectrum are well described, far less is known about age-specific structural variation and its functional implications.

Proteins, particularly multi-functional proteins such as Protein C, exert their functions through protein-protein interactions (PPIs) in complex with other proteins and co factors (16, 17). Changes in PPIs modulate the functions of individual protein components within these complexes, a phenomenon known as moonlighting (18). Interactomics is the study of PPIs in biological systems, and has begun to shed light on the intricate and complex functioning of living organisms (16). Co-immunoprecipitation (coIP), the antibody mediated pull-down of a protein of interest and its interaction partners, coupled to mass spectrometry-based proteomics is a common technique for investigating PPIs (19, 20).

Here, we aimed to characterise age-related differences in Protein C function by investigating differences in protein interaction partners of Protein C. We performed coIP on pooled plasma from healthy individuals within specific age ranges from neonate to adult. Proteins differentially enriched between coIPs and controls were identified in each age group, and differences between age groups were examined. This study sheds further light on the complex age associated changes of the haemostatic system, and suggests the intricate interplay of protein-protein networks has changing functions throughout ageing, exemplified in this instance by Protein C.

Methods

Sample Collection:

Sample collection was performed as part of the HappiKids study (21), as outlined below regarding participant numbers, pools and age groups.

Participant Recruitment:

Healthy individuals attending Royal Children's Hospital (RCH), Melbourne, Australia, for minor day procedures who met eligibility were recruited for the study. Neonate samples were collected at the Royal Women's Hospital (RWH) from healthy babies with consent from the parents. Consent and eligibility were confirmed by interview prior to sample collection.

Samples collection and preparation:

Blood samples were collected by anaesthetists from paediatric patients prior to the administration of anaesthetic. Neonatal samples were collected by our specialist collectors. S-Monovette citrate tubes with a ratio of 1 part sodium citrate to 9 parts blood were used for sample collection and thereafter transported to the Melbourne Children's Campus biobanking facility for centrifugation and aliquoting. Samples were processed within 4 hours of collection. Tubes were spun at 3000 rpm for 10 minutes and PPP fraction was removed, aliquoted and stored at -80 C until used.

A total of 180 healthy participants were recruited into the study, separated into three pools, each consisting of 6 age groups with 10 individuals per group (5 males and 5 females). Participant plasma samples were allocated to one of 6 age groups: Neonates, 28 days to 1 year, 1 to 5 years, 6 to 10 years, 11 to 17 years and adults.

Ethics approval:

All procedures were approved by the Royal Children's Hospital Human Research Ethics Committee HREC Reference Number: 34183, 32287 B HREC/14/RCHM/61

Co-ImmunoPrecipitation (coIP)

Dynabeads Protein G Immunoprecipitation Kit (Thermo Fisher, Australia) magnetic beads, conjugated with mouse anti-human PC monoclonal antibody Clone 067 (IMSAHU067CLY, Innovative Research, USA) were utilised for coIP. The antibody was conjugated with the Dynabeads by incubation for 2.5 hours at room temperature. Following incubation and thrice washing with 1 mL Phosphate Buffered Saline with 0.02% Tween 20 (PBST) and 1 mL conjugation buffer, Pierce BS3 crosslinker (Thermo Scientific) was used to crosslink the 067 antibody with the magnetic beads for 1 hour with rotation. Reactions were quenched for 1 hour with the addition of 12.5 μ L of 1M Tris at pH 7.6. Following incubation, 200 μ L of 0.2 M Glycine at pH 2.6 was added for a 1-minute incubation to elute any non-specifically bound material. The magnetic beads conjugated with the 067 antibody were subsequently washed thrice with 1 mM CaCl_2 PBST. Plasma, diluted 1:1 with PBS and brought to a final concentration of 1 mM CaCl_2 , was subsequently introduced to antibody-conjugated magnetic beads and incubated for three hours at 4°C with gentle agitation to allow capture of Protein C by the 067 antibody. After incubation, the Dynabeads and any bound proteins were separated from the plasma via magnet, and the plasma supernatant removed. Beads were washed thrice with 1mM CaCl_2 PBST, 5-minute incubation with mild agitation, with the supernatant removed after separation of the beads from solution via magnet. After washing, proteins bound to the antibody conjugated beads were eluted using elution buffer with 2% sodium dodecyl sulfate (SDS), 1M Tris (tris(hydroxymethyl) aminomethane) pH 7.6, 0.5M EDTA (Ethylenediaminetetraacetic acid), reverse osmosis water (RO H_2O) and incubation at 50°C for three minutes, repeated three times. The eluates were then aliquoted and stored at - 80 C for mass spectrometry analysis.

Mass Spectrometry (MS)

MS sample preparation

For protein-C immunoprecipitation eluates samples were prepared using the filter-assisted sample preparation (FASP) protocol as described previously (22). Resultant peptide digests were vacuum centrifuged to dryness, and peptides reconstituted in 0.1% formic acid (FA), 2% acetonitrile (ACN) in preparation for LC-MS/MS.

MS Acquisition

Mass spectrometry analysis was performed using the timsTOF pro MS (Bruker). Peptides were separated on a C18 fused silica column (inner diameter 75 μ m, OD 360 μ m $\times$ 15 cm length, 1.6 μ m C18 beads) packed into an emitter tip (Aurora Elite, IonOpticks) using a custom nano-flow HPLC system (Thermo Ultimate 300 RSLC Nano-LC, PAL systems CTC autosampler). The HPLC was coupled to a timsTOF Pro equipped with a CaptiveSpray source. Peptides were loaded directly onto the column at a constant flow rate of 600 nL/min with buffer A (99.9% Milli-Q water, 0.1% FA) and eluted with a 30-min linear gradient from 2 to 34% buffer B (90% ACN, 0.1% FA). The timsTOF Pro (Bruker) was operated in diaPASEF mode using Compass Hystar 5.1. The settings on the TIMS analyser were as follows: Lock Duty Cycle to 100% with equal accumulation and ramp times of 100 ms, and 1/K0 Start 0.6 V $\cdot$ /cm² End 1.6 V $\cdot$ s/cm², Capillary Voltage 1400V, Dry Gas 3 l/min, Dry Temp 180°C. The DIA methods were set up using the instrument firmware (timsTOF control 2.0.18.0) and included two windows in each diaPASEF scan across 16 $\times$ 25 m/z precursor isolation windows (32 windows) defined from m/z 400 to 1,200, with 1 Da overlap, and CID collision energy ramped stepwise from 20 eV at 0.8 V $\cdot$ s/cm² to 59eV at 1.3 V $\cdot$ s/cm².

Database searching was performed using Spectronaut software (v.19.0) against the human-reviewed databases (UniProt downloaded July 2022) using the BGS standard settings, including cross-run normalisation but no imputation. Cysteine carbamidomethylation was included as a fixed modification, and variable modifications were set as N-terminal acetylation and methionine oxidations. The FDR and PEP cutoff were set to less than 1% at the peptide and

protein levels, and a minimum length of seven amino acids for peptides was specified. Enzyme specificity was set trypsin proteases with a maximum of two missed cleavages.

Statistical Analysis of Protein C interactome

Data processing and analysis of the Spectronaut output were performed using R software (v.4.4.1). Protein groups included in the statistical analysis were derived from proteotypic peptides and were identified in 67% of samples in at least one group were kept. No imputation was applied, except for proteins entirely missing in one group but present in others to allow for statistical analysis. For these cases, missing values were replaced with random numbers drawn from a normal distribution, with a 1.8 standard deviation downshift and a width of 0.3. A total of 496 proteins were analysed, and their intensities were log2-transformed. Data normalization was performed using the RUV-IIIc method (v. 1.0.19) [4], utilizing 236 empirically chosen proteins as negative controls, specifically those that remained invariant across all conditions (P-value > 0.5). Multivariate analysis, including Principal Component Analysis (PCA), was employed to identify any potential outliers. Pair-wise comparisons were conducted using the limma package (v. 3.60.3) in R. Adjustments for multiple comparisons were applied using the Benjamini-Hochberg procedure to control the false discovery rate. A protein was deemed significantly differentially expressed if its false discovery rate (FDR) was 5% or lower.

Functional enrichment analysis was performed on the sets of differentially expressed proteins to identify over-represented biological pathways and functions. This analysis utilized the databases Reactome (23) and Gene Ontology (GO) (24, 25), employing the clusterProfiler (v. 4.12.2) R-package. Visualization of the data and the results of the analyses was performed using ggplot2 (v. 3.5.1).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (26) partner repository with the dataset identifier PXD077907 (Username: reviewer_pxd @ebi.ac.uk, Password: rQ11IGGcK4j).

STRING protein network analysis

Protein network analysis was performed using STRING v12.0 (<https://string-db.org/>) (27), inputting the full list of significantly enriched proteins from each age group, and using default settings including known interactions (from curated databases and experimentally determined), predicted interactions (neighbourhood in the genome, gene fusions, co-occurrence across genomes) and textmining (co-mention in Pubmed abstracts, including putative homologs mentioned together in other organisms), co-expression (including putative homologs co-expressed in other organisms) and protein homology.

Results

Immunoprecipitation (IP) enrichment of Protein C and its co-interacting proteins (coIP) was successfully performed in pooled plasma samples in six groups spanning neonates through to adults. A non-redundant total of 496 plasma proteins were included in the analysis post-reproducibility filtering and pre-processing.

Non-redundant statistically significant proteins were identified as enriched in pairwise comparison in each of the six age groups, where the coIP condition and its unconjugated control were directly compared (Fig. 1). These significant proteins were considered to engage in PPI with Protein C based on an adjusted p-value cut-off of 0.05. Enriched proteins unique to each age group or jointly between age groups were identified. The number and identities of enriched and depleted proteins varied across age groups, with the highest number of Protein C PPI partners observed in the 11–16 year-old group. We did not observe that the number of PPI identified followed any trends with age. Partial Least Squares Discriminant Analysis (PLS-DA) showed clear separation of enrichments between all age groups, with some overlap seen between the 11–16 year-old and adult groups (Figure S1).

The fold-change increase in Protein C was not consistent across age groups through the six paired comparisons. This was consistent with the relative enrichment of PROC in each age-group plasma pool. Protein C enrichment from plasma was lowest in neonates, followed by the 1 month-1 year-old group (Figure S2). Strikingly, in the neonates and the 1 month-1 year-old group, Protein C was not the most highly enriched protein in the coIP treatment. In all other groups Protein C had the highest fold-change of all enriched proteins (Fig. 1, Table 1).

Overall, we observed clear enrichment of Protein C in all conditions at levels consistent with expectations for plasma given the extreme dynamic range of this biofluid and the persistence of abundant, non-specific plasma protein binders.

Table 1

The five most highly enriched proteins in the colP treatment as compared to controls in each age group, listed in order of log2 fold-change increase (FC). The 'bait' of Protein C is bolded. Right hand column indicates whether the protein was found to be significantly enriched only in that age group (unique) or not.

Protein Accessions	Protein Descriptions	Gene	FC	P Adj.	unique y/n
Neonate					
P01137	Transforming growth factor beta-1 proprotein	TGFB1	5.19	7e-3	y
P62851	40S ribosomal protein S25	RPS25	4.85	7e-3	y
P29508	Serpin B3	SERPINB3	4.44	2e-2	y
P14555	Phospholipase A2, membrane associated	PLA2G2A	4.13	4e-3	n
P04070	Vitamin K-dependent Protein C	PROC	4.09	3e-2	n
1 month – 1 year					
Q15582	Transforming growth factor-beta-induced protein ig-h3	TGFB1	6.01	8e-4	n
P04070	Vitamin K-dependent Protein C	PROC	5.51	8e-3	n
P63104	14-3-3 protein zeta/delta	YWHAZ	5.37	4e-2	y
Q9Y6R7	IgGfc-binding protein	FCGBP	4.67	2e-5	n
Q96QZ7	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1	MAGI1	4.49	5e-3	y
1–5 years					
P04070	Vitamin K-dependent Protein C	PROC	8.28	1e-4	n
O95810	Caveolae-associated protein 2	CAVIN2	6.61	1e-3	y
P13224	Platelet glycoprotein Ib beta chain	GP1BB	5.62	5e-2	y
Q13103	Secreted phosphoprotein 24	SPP2	5.13	1e-3	n
Q13201	Multimerin-1	MMRN1	5.09	4e-3	y
6–10 years					
P04070	Vitamin K-dependent Protein C	PROC	7.42	1e-4	n
P03952	Plasma kallikrein	KLKB1	6.88	1e-3	y
P12259	Coagulation factor V	F5	5.39	5e-3	y
O75636	Ficolin-3	FCN3	5.05	1e-3	n
O00187	Mannan-binding lectin serine protease 2	MASP2	3.31	4e-3	n
11–16 years					
P04070	Vitamin K-dependent Protein C	PROC	8.81	1e-4	n
P19652	Alpha-1-acid glycoprotein 2	ORM2	7.49	2e-5	n
P02760	Protein AMBP	AMBP	6.95	6e-9	y
O75223	Gamma-glutamylcyclotransferase	GGCT	5.62	3e-2	y
P29622	Kallistatin	SERPINA4	5.27	6e-4	y
Adult					
P04070	Vitamin K-dependent Protein C	PROC	7.24	7e-4	n
Q96N96	Spermatogenesis-associated protein 13	SPATA13	5.34	1e-2	n
P21333	Filamin-A	FLNA	4.46	1e-2	n
P14618	Pyruvate kinase PKM	PKM	4.25	7e-4	n
A0A0C4DH35	Probable non-functional immunoglobulin heavy variable 3–35	IGHV3-35	4.14	7e-4	y

Unique PPIs based on age were detected in all pairwise comparisons between colP and unconjugated controls (Fig. 2). Protein C was the only protein identified as statistically significantly enriched across the six pairwise comparisons. Only three proteins were significantly enriched in 5/6 age pairwise comparisons. These were Fibulin-1, enriched in all groups except neonates, and Clusterin and Immunoglobulin heavy constant mu, which were enriched in all but the 6–10-year-old group (full data set at ProteomeXchange: identifier PXD077907).

The highest number of enriched proteins in the Protein C IP samples were seen in the 10–16-year-old group (54 proteins), which also had the highest number (33 proteins) and highest proportion (61.1%) of uniquely enriched proteins. The neonate group had the second highest proportion of uniquely enriched proteins (55.6%), while the 5–10-year-old group had the lowest number of enriched proteins (7), and the 1 month-1 year old group had the lowest proportion of uniquely enriched proteins (11.2%) (Fig. 2, Table S1).

STRING Interaction Analysis:

STRING v12.0 (<https://string-db.org/>) (27) was used to gain biologically meaningful insight into Protein C and PPI partners. Significantly enriched proteins were inputted for each age group, and distinct interaction networks were predicted for each age group (Fig. 4, Table 2). Unsurprisingly, these were found to show increasing complexity with increasing number of interactors identified.

Table 2
Number of nodes and edges identified in STRING analysis of Protein C and co-precipitating proteins. Edges represent the predicted functional associations and nodes represent proteins.

number of nodes:	Neonate	1 month – 1 year	1–5 years	6–10 years	11–16 years	Adult
	26	15	26	7	50	18
number of edges:	43	9	59	10	160	22

Outside of providing a visual representation of predicted protein networks, STRING can also predict proteins that will interact directly with a nominated protein based on known and predicted interactions. Direct Protein C interactors predicted by STRING analysis in each age group were identified (Fig. 3, Table 3). Interestingly, while the largest number of direct interactors were predicted for the 11–16 year-old group, the group having the largest number of enriched interactors, the number of predicted direct interactors did not correspond with the number of significantly enriched proteins across the age groups.

Table 3
Direct Protein C interactors predicted by STRING analysis among the enriched proteins in each age group. Proteins present in multiple groups are shown in *italics*, proteins among the top 5 most highly enriched in each age group are **bolded**.

Neonate	MASP1	SERPINA5										
1 month – 1 year	CD5L	FCN3										
1–5 years	APOH	CD5L	CFH	FCN3	GP1BB	SERPINF2	SPP2					
6–10 years	F5	FCN3	KLKB1	MASP2								
11–16 years	AMBp	APOH	CD5L	CPB2	F10	F13A1	F9	MASP1	MASP2	PF4	SPP2	TGM1
Adult	CD5L	FCN3										

Notably, a number of proteins were both predicted to be direct Protein C interactors and among the most highly enriched proteins. These were GP1BB (platelet glycoprotein Ib beta chain) and SPP2 (secreted phosphoprotein 24) in the 1–5 year-old group, AMBP (alpha-1-microglobulin/ bikunin precursor) in the 11–16 year-old group, and all in the 6–10 year-old group. Only in the 6–10 year-old group were all proteins predicted by STRING to be direct interactors among the most highly enriched.

Pathway Analysis:

Significantly enriched proteins were searched against the Gene Ontology (GO) database (24, 25) and the Reactome database (23) to identify over-represented biological pathways and functions. 23 GO pathways and 102 Reactome pathways were found to be significantly enriched based on adjusted p-value in the colPs across all age groups (see Table S2 and Table S3 for full pathway analysis details).

GO biological process (GO-BP) pathways enriched in ColPs showed differences and similarities between age groups. The most significant (based on adjusted p value, gene ratio and count) enriched pathways in the neonates and 6–10 year groups are very similar to one another and both similar to the 11–16 year old groups, with most enriched pathways including those involved in wound healing, coagulation and haemostasis. The 1 month – 1 year group is most similar to adults, with more pathways involving immunity more highly enriched (Fig. 4). GO biological process (GO-BP) pathways enriched in ColPs strictly containing PROC in the gene list were limited to processes associated with haemostasis, coagulation and healing, while processes enriched that did not necessarily include PROC in the gene list included pathways associated with immune responses (Fig. 4).

Differences and similarities were likewise seen in Reactome pathways between all age groups. The top 5 Reactome pathways enriched in IP samples were predominantly connected to platelet activation and degranulation, fibrin clot formation, the complement cascade, and cell signalling (Table S2).

Many of the most significantly enriched Gene Ontology (GO) pathways identified were found to be significantly enriched across most or all of the age groups. However, in most cases, different proteins were found to be driving enrichment of these pathways. In many age groups, proteins found to either be among the most highly enriched and/or predicted to be direct Protein C interactors feature prominently in the enriched pathways (Table 4). For example, in the 1–5 year-old group, GP1BB (Platelet glycoprotein Ib beta chain, a component of the von Willebrand factor (vWF) receptor complex that is necessary for platelet formation and activation (28)) was found to participate in many of the most significant pathways identified – unsurprisingly, those associated with coagulation – as well as being the third most highly enriched protein in the colP and being predicted to be a direct interactor of Protein C. This protein was

not found to be significantly enriched in any other group. In the 6–10 year-old group, the proteins predicted to be direct interactors were the top 5 most highly enriched and participated in almost all of the most significantly enriched pathways.

Interestingly, this was the only group in which pathways associated with immune responses were not significantly enriched. In the 11–16 year-old group, many of the predicted direct interactors feature in many of the enriched pathways. However, none of these were among the most highly enriched proteins. FBLN1 (Fibulin-1) and CLU (Clusterin), which were identified in 5/6 age group colPs, but were not among the most highly enriched or predicted direct interactors, contribute to most of the more significantly enriched pathways in most age groups. These will be discussed in more detail below.

Table 4

Gene names of proteins driving significant highly enriched GO (biological process) pathways between age groups. Proteins among the 5 most highly enriched protein (PROC) is shown in red.

Biological process (GO BP)	neonates	1 mnth-1 yr	1–5 years	6–10 years	11–16 years
wound healing GO:0042060	<i>TGFB1</i> /APCS/PROC/CTSG/CD36/CD9/ TLN1	PROC/FLNA/ FBLN1	APCS/ APOH / PROC/ SERPINF2 / GP1BB /FBLN1/ ACTB/MMRN1/ AJUBA	KLKB1 /PROC/ F5 /FBLN1	F13A1 / F10 / APOH / I PRDX2/ F9 / CPB2
blood coagulation GO:0007596	PROC/CTSG/CD36/CD9/ TLN1	PROC/FLNA/ FBLN1	APOH/PROC/ SERPINF2 / GP1BB /FBLN1/ACTB/ MMRN1	KLKB1 /PROC/ F5 /FBLN1	F13A1 / F10 / APOH / I PRDX2/ F9 / CPB2
Coagulation GO:0050817	PROC/CTSG/CD36/CD9/ TLN1	PROC/FLNA/ FBLN1	APOH/PROC/ SERPINF2 / GP1BB /FBLN1/ACTB/ MMRN1	KLKB1 /PROC/ F5 /FBLN1	F13A1 / F10 / APOH / I PRDX2/ F9 / CPB2
Hemostasis GO:0007599	PROC/CTSG/CD36/CD9/ TLN1	PROC/FLNA/ FBLN1	APOH/PROC/ SERPINF2 / GP1BB /FBLN1/ACTB/ MMRN1	KLKB1 /PROC/ F5 /FBLN1	F13A1 / F10 / APOH / I PRDX2/ F9 / CPB2
reg. body fluid levels GO:0050878	PROC/CTSG/CD36/CD9/ TLN1	PROC/FLNA/ FBLN1	APOH/PROC/ SERPINF2 / GP1BB /FBLN1/ACTB/ MMRN1	KLKB1 /PROC/ F5 /FBLN1	F13A1 / F10 / APOH / I PRDX2/ F9 / CPB2
humoral immune response GO:0006959	IGHM/CTSG/CLU/ MASP1	CD5L / FCN3 / JCHAIN/IGHM/ IGHA1/CLU/ IGHA2	CD5L / FCN3 / JCHAIN/IGHM/ IGHA1/ CFH /CLU	MASP2 / FCN3	MASP2 / CD5L / IGHM/ PF4 /CLU/ MASP1 /IGHA2
complement activation GO:0006956	IGHM/CLU/ MASP1	CD5L / FCN3 / IGHM/IGHA1/ CLU/IGHA2	CD5L / FCN3 /IGHM/IGHA1/ CFH /CLU	MASP2 / FCN3	MASP2 / CD5L / IGHM/CLU/ MASP1 /IGHA2
leukocyte mediated immunity GO:0002443	<i>TGFB1</i> /IGHM/CTSG/CLU	IGHM/IGHA1/ ARG1/CLU/ IGHA2	IGHM/IGHA1/ ARG1/CLU	n/a	IGHV3-74/ MASP2 /IGHM/ CLU/PRDX1/ IGHA2/IGHD
lymphocyte mediated immunity GO:0002449	<i>TGFB1</i> /IGHM/CLU	IGHM/IGHA1/ ARG1/CLU/ IGHA2	IGHM/IGHA1/ ARG1/CLU	MASP2	IGHV3-74/ MASP2 /IGHM/ CLU/PRDX1/ IGHA2/IGHD
adaptive immune response GO:0002460	<i>TGFB1</i> /IGHM/CLU	IGHM/IGHA1/ ARG1/CLU/ IGHA2	IGHM/IGHA1/ ARG1/CLU	MASP2	IGHV3-74/ MASP2 /IGHM/ CLU/IGHA2/IGHD

Discussion

We investigated age-related changes in the interactome and PPI of Protein C, a key component of the haemostatic system. Important differences, at the individual protein level and at the pathway level, were found across the age range, particularly in neonates and infants. Developmental changes in the coagulation system are known (9, 11). Different antigenic and functional levels of key coagulant and anticoagulant proteins, including Protein C, across the age range point towards overall differences in the functioning in the coagulation system in neonates and children, as does the reduced risk of both bleeding and clotting experienced by children (9, 10). Identification of interaction partners within the coagulation system can help reconcile these differences.

Differences in Protein C immunoprecipitation enrichment between the age groups, both at the level of the individual interacting proteins identified and at the pathway level, warrant further discussion, particularly in the younger age groups. The fold-change increase of Protein C was lowest in the neonate group, followed by the 1 month – 1 year-old group. This may be partly due to the naturally lower abundance of Protein C in neonates and in the 1 month-1-year-old group (40–50% that of adult levels) (9). However, the values reported here are log2 fold change. A log2 fold-change of 4 compared to log2 fold-change of 8 therefore represents a comparative enrichment considerably lower than would be explained by availability due to abundance. This may indicate that the binding of Protein C by clone 067 antibody varies across the age range and is weakest in the neonate group. This may be due to differential post-translational modifications or conformational differences in the protein in infants or may be caused by other PPIs interfering with antibody binding, or a combination of both.

A combination of Gene Ontology (GO) pathway analysis and prediction of interaction networks (STRING analysis) can help shed light on the biological relevance of the interactors and interactions identified in our CoIPs. Many of the pathways enriched in the Protein C CoIPs of all age groups were related to wound healing, coagulation and immune responses, as would be expected for plasma protein interactomes of a protein of the coagulation cascade. Considerable overlap exists between proteins involved in the most significantly enriched pathways, proteins most significantly enriched, and those predicted to be direct interactors, suggesting these individual proteins may be of interest in relation to Protein C. In the neonate group, four proteins were enriched with higher fold-change increases than Protein C. Outside of the 1 month – 1 year group, which had one protein more highly enriched, all other groups saw Protein C enriched with the highest fold-change of all enriched proteins, as would be expected for an IP enrichment using an anti-Protein C antibody. Proteins with increased abundance in Protein C co-IP as compared to control in the neonate group, in order of highest fold-change, were: Transforming growth factor β 1 (TGF- β 1), 40S ribosomal protein S25 (RPS25), serine protease inhibitor B3 (SERPINB3), and Phospholipase A2 Group IIA (PLA2-IIA). Of these, the first three were enriched only in the neonate group and PLA2-IIA was enriched in neonates and the 10–16-year-old group.

The cytokine TGF- β 1 is involved in regulation of several cellular processes including cell proliferation, differentiation, wound healing, and immune response (29). TGF- β 1 regulates multiple events in bone marrow, including quiescence of hematopoietic stem cells (30). TGF- β 1 is highly expressed by platelets and megakaryocytes, and has been shown to regulate megakaryopoiesis by downregulating thrombopoietin production in liver (31). Furthermore, TGF- β has been implicated in aging of the haematopoietic system (30).

Phospholipase A2, Group IIA (PLA2-IIA) is a secretory calcium-dependent phospholipase A2 with implications in host antimicrobial defence, inflammatory response, and tissue regeneration. PLA2-IIA is constitutively expressed in platelets and endothelial cells, among others, and primarily targets (hydrolyses) extracellular phospholipids (32–34). Given the known involvement of phospholipids in Protein C activation (35–37), the association of PLA2-IIA and Protein C in two age groups is interesting. PLA2-IIA binds to and activates integrins ITGA5:ITGB3, ITGA4:ITGB1 and ITGA5:ITGB1, receptors for fibrillin, fibronectin and fibrinogen (38, 39). Interestingly, Fibulin-1 (FBLN1), which was found to be enriched in all groups except neonates and featured strongly in many highly enriched pathways, mediates platelet adhesion via binding fibrinogen [49]. Age related differences in PLA2-IIA interaction have been reported, and it has been shown to be important in the transition from the neonatal immune system to adult (40). Its increased association with Protein C in the neonate group may indicate an increased role for PLA2-IIA in the haemostatic system of neonates. Interestingly, direct interactions between TGF- β 1, PLA2-IIA, CD36 and CD9 (involved in many of the highly enriched pathways) in the neonate group are predicted in our STRING analysis (Fig. 3).

RPS25 is involved in alternative protein translation initiation routes (41), has a potential role in inflammatory responses (42) and tumour suppression via the p53 pathway (43, 44). RPS25 was found to be down-regulated in acute myocardial infarction (AMI) in endothelial cells (45) and in megakaryocytes (46). This may infer a protective role for this protein being more strongly associated with Protein C in neonates. However, ribosomal proteins are not considered a normal component of the plasma proteome, and their association with Protein C in neonates may warrant further investigation.

SERPINB3 is involved in cell motility, migration and differentiation, angiogenesis, and is important in regulating differentiation of the squamous epithelium (47, 48). SERPINB3 has been identified as a biomarker in a number of conditions (48–51), and has been associated with wound healing (52). Higher SERPINB3 expression was found in infant skin samples as compared to adult (47). SERPINB3 has been associated with upregulation of TGF β 1 (48, 53), which is interesting in light of the current study, as TGF β 1 was also found to have increased interaction with Protein C in our neonate samples. Studies have shown a correlation between increased SERPINB3 levels and increased TGF β 1 expression in disease states (54), but their association in healthy individuals, particularly in paediatric populations, has not been investigated, nor has their association with Protein C.

In the 1 month – 1 year group TGFBI (Transforming growth factor-beta-induced protein ig-h3) was enriched with higher fold change than Protein C. This protein was also significantly enriched in the 1–5 year-old and 11–16 year-old groups. Involved in angiogenesis, wound healing and inflammation, TGFBI is known to have many interaction partners in extracellular matrix, including collagen, fibronectin, laminin, and glycosaminoglycans (55), but interacting proteins in blood or plasma have not been reported.

Sequence alignment (BLASTp (56)) performed between Protein C and these more highly enriched proteins revealed no significant homology, indicating these were likely not interacting directly with the clone 067 antibody Protein C binding site, and suggesting interactions with Protein C and other interacting

proteins. Increased interaction of these proteins with Protein C in neonates and young infants underscores the complex and varying nature of the coagulation system across the age range.

Other individual proteins of interest in the neonate group are the predicted direct interactors SERPINA5 (Protein C inhibitor; enriched only in the neonate group) and MASP1 (Mannan binding lectin serine peptidase 1), direct interactor in neonate and 11–16 year-old groups). SERPINA5 can act as a procoagulant and pro-inflammatory factor by inhibiting both APC activity (57) and generation by the thrombin/thrombomodulin complex (58). However, SERPINA5 can also act as an anticoagulant factor by inhibiting factor Xa, thrombin (59), plasma kallikrein and factor XI (60), with a stronger anticoagulant activity observed with reduced Protein C levels (58). This, and the stronger association of Protein C and SERPINA5 in neonates compared to other groups, may help to explain why lower Protein C levels observed in neonates do not lead to increased thrombosis, with SERPINA5 contributing more to anticoagulant activity at this stage of development.

MASP1 and MASP2, both predicted to interact directly with Protein C in 2 age groups, are mannose-binding lectin-associated serine proteases involved in triggering of the lectin pathway of the complement system (61). FCN3 (Ficolin-3), predicted to be directly interacting with Protein C in 4 age groups, is a soluble, calcium-dependent defence protein with lectin-like activity, which acts as a pattern recognition receptor that activates the MASP1 serine protease and initiates the proteolytic cascade of the lectin complement pathway (62). Interestingly, either FCN3, MASP1 or MASP2 were found in all groups and were predicted by STRING analysis to be direct interactors of Protein C. This may suggest a stronger interplay between Protein C and the complement system, especially the lectin pathway, than previously investigated, and again highlights the changing roles of proteins within pathways throughout development.

CD36 (Platelet glycoprotein 4) features strongly in neonate pathway analysis, is involved in angiogenesis (63), is a receptor for thrombospondin (enriched in 11–16 year-old group), and is a known interactor of CD5L (64, 65) (predicted direct Protein C interactor in 3/6 groups). CD5L (CD5 antigen-like molecule) was predicted to be directly interacting with Protein C in 4 age groups. Mainly expressed by macrophages, this secreted protein acts as a pattern recognition molecule, regulates the expression of pro-inflammatory genes by regulation of lipid synthesis, and inhibits apoptosis in macrophages, promoting macrophage survival in atherosclerosis (66–69).

SPP2 (Secreted phosphoprotein 2, predicted direct Protein C interactor in 2 age groups), is a liver-derived protein known to have a role in bone growth and turnover, to activate intracellular signalling pathways and to bind cytokines of the TGF- β superfamily (70). TGF- β 1 was significantly enriched in (and only in) our neonate group, but SPP2 was not. In GO pathway analysis, SPP2 was found to be involved in peptidase regulation, and Reactome pathways show it to be involved with platelet activation and degranulation. Interestingly, in the neonate group, TGF- β 1 contributes to platelet degranulation pathways (Reactome analysis), and peptidase regulation (GO pathways) (see supplementary).

CLU (Clusterin) is an extracellular chaperone (71) enriched in 5/6 age group CoIPs, and was found to participate in many immune related pathways. Given its role in clearance of misfolded proteins, this may have been present and binding in our samples as an artifact rather than a biologically functional interactor of Protein C. However, as a multifunctional protein with cytoprotective functions (72), as Protein C has, CLU and Protein C may function together in an as yet undescribed protective protein complex.

At all levels of analysis, the data point to significant differences in the interactome of Protein C with age. This is in line with current knowledge about the changes in the plasma proteome with age, and underlines the complex, changing and multifaceted roles of many proteins involved in the coagulation and immune systems. Differences seen between age groups, despite all samples being treated the same, highlight the fundamental differences in the plasma proteome and haemostatic system between children and adults.

Conclusion

We present the first comprehensive investigation of Protein C PPIs, as determined by colP-MS, in age groups spanning from neonates to adults. Differences were found between all age groups in interacting partners of Protein C and the pathways they are involved in, with the most pronounced differences seen in the youngest cohorts. The findings of this study highlight global differences in the haemostatic system across normal development. Differences in protein structure and function, modifications, interactions and biological pathways in neonates and younger children are important to understand as these may lead to a better understanding of ageing processes and improve health outcomes for children and for adults. Protein C is known to have functions outside of its anticoagulant role [56]. As identified in this study, Protein C interacts with multiple proteins, many of them not directly related to the coagulation system, and these interaction partners change with age. This may indicate a more complex suite of functions for Protein C than previously understood. Interactomics experiments such as this may allow for a more holistic understanding of developmental haematology. Furthermore, this work underscores the difficulties encountered when working with small sample volume availability (problematic when collecting blood samples from infants) and the problems associated with studying native plasma proteins as opposed to recombinantly produced and highly purified protein samples. Further work will be required to develop interactome maps of the developing haemostatic system, which may require improvements in purification and analysis techniques. Finally, our findings provide cause for consideration for clinicians giving protein concentrates to children. Our results, suggest different off-target effects could occur, compared to adults, and further research of this potential phenomenon is required.

Abbreviations

ACN
Acetonitrile
APC
Activated Protein C
colP

Co-immunoprecipitation
EDTA
Ethylenediaminetetraacetic acid
FASP
Filter-assisted sample preparation
FA
formic acid
FDR
False discovery rate
GO
Gene Ontology
LC
MS/MS-Liquid chromatography-mass spectrometry/mass spectrometry
PLS
DA-Partial least squares discriminant analysis
PPIs
Protein-protein interactions
PCA
Principal component analysis
PEP
Posterior error probability
RCH
Royal Children's Hospital
RO H₂O
Reverse osmosis water
RWH
Royal Women's Hospital
SDS
Sodium dodecyl sulfate
Tris
Tris(hydroxymethyl) aminomethane)

Declarations

Ethics approval and consent to participate:

All procedures were approved by the Royal Children's Hospital Human Research Ethics Committee HREC Reference Number: 34183, 32287 B HREC/14/RCHM/61

Consent for publication:

The manuscript does not contain any individual person's data in any form and does not require consent for publication.

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Author Contribution

SC, VK, PM and CA conceived the study. VK coordinated sample collection and allocation. SC and KYC performed CoIP and sample preparation. SJE and LFD performed LC-MS/MS experiments and data collection and curation. JMY performed statistical analysis of MS data and assisted with figure preparation. SC, PM, CA and SJE analysed and interpreted data. SC wrote the first and subsequent manuscript drafts. All authors reviewed and approved the final manuscript.

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Data Availability

References

1. Foster DC, Yoshitake S, Davie EW. The nucleotide sequence of the gene for human protein C. *Proceedings of the National Academy of Sciences*. 1985;82(14):4673-7.
2. Mesters RM, Houghten RA, Griffin JH. Identification of a sequence of human activated protein C (residues 390–404) essential for its anticoagulant activity. *J Biol Chem*. 1991;266(36):24514–9.
3. Strickland DK, Kessler CM. Biochemical and functional properties of protein C and protein S. *Clin Chim Acta*. 1987;170(1):1–23.
4. Zhao R, Lin H, Bereza-Malcolm L, Clarke E, Jackson CJ, Xue M. Activated Protein C in Cutaneous Wound Healing: From Bench to Bedside. *Int J Mol Sci*. 2019;20(4):903.
5. Fair D, Marlar R. Biosynthesis and secretion of factor VII, protein C, protein S, and the Protein C inhibitor from a human hepatoma cell line. *Blood*. 1986;67(1):64–70.
6. Kisiel W, Human Plasma Protein C. ISOLATION, CHARACTERIZATION, AND MECHANISM OF ACTIVATION BY α -THROMBIN. *J Clin Invest*. 1979;64(3):761–9.
7. Berry LR, Van Walderveen MC, Atkinson HM, Chan AKC. Comparison of N-linked glycosylation of protein C in newborns and adults. *Carbohydr Res*. 2013;365:32–7.
8. Weiss P, Soff GA, Halkin H, Seligsohn U. Decline of proteins C and S and factors II, VII, IX and X during the initiation of warfarin therapy. *Thromb Res*. 1987;45(6):783–90.
9. Attard C, van der Straaten T, Karlaftis V, Monagle P, Ignjatovic V. Developmental hemostasis: age-specific differences in the levels of hemostatic proteins. *J Thromb Haemost*. 2013;11(10):1850–4.
10. Monagle P, Barnes C, Ignjatovic V, Furmedge J, Newall F, Chan A et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. 2006;95(02):362–72.
11. Ignjatovic V, Lai C, Summerhayes R, Mathesius U, Tawfilis S, Perugini MA, et al. Age-Related Differences in Plasma Proteins: How Plasma Proteins Change from Neonates to Adults. *PLoS ONE*. 2011;6(2):e17213.
12. Toulon P, Berruyer M, Brionne-François M, Grand F, Lasne D, Telion C, et al. Age dependency for coagulation parameters in paediatric populations. *Thromb Haemost*. 2016;116(07):9–16.
13. Manco-Johnson MJ, Spedale S, Peters M, Townsend SF, Jacobson LJ, Christian J, et al. Identification of a Unique Form of Protein C in the Ovine Fetus: Developmentally Linked Transition to the Adult Form. *Pediatr Res*. 1995;37(3):365–72.
14. Greffe BS, Manco-Johnson MJ, Marlar RA. Molecular Forms of Human Protein C: Comparison and Distribution in Human Adult Plasma. *Thromb Haemost*. 1989;62(07):902–5.
15. Greffe BS, Marlar RA, Manco-Johnson MJ. Neonatal protein C: Molecular composition and distribution in normal term infants. *Thromb Res*. 1989;56(1):91–8.
16. Iacobucci I, Monaco V, Cozzolino F, Monti M. From classical to new generation approaches: An excursus of -omics methods for investigation of protein-protein interaction networks. *J Proteom*. 2021;230:103990.
17. Alberts B. The Cell as a Collection of Protein Machines: Preparing the Next Generation of Molecular Biologists. *Cell*. 1998;92(3):291–4.
18. Jeffery CJ. Protein moonlighting: what is it, and why is it important? *Philosophical Trans Royal Soc B: Biol Sci*. 2017;373(1738).
19. Lin JS, Lai EM. Protein-Protein Interactions: Co-Immunoprecipitation. *Methods Mol Biol*. 2017;1615:211–9.
20. Gnanasekaran P, Pappu HR. Affinity Purification-Mass Spectroscopy (AP-MS) and Co-Immunoprecipitation (Co-IP) Technique to Study Protein-Protein Interactions. *Methods Mol Biol*. 2023;2690:81–5.
21. Hoq M, Karlaftis V, Mathews S, Burgess J, Donath SM, Carlin J, et al. A prospective, cross-sectional study to establish age-specific reference intervals for neonates and children in the setting of clinical biochemistry, immunology and haematology: the HAPPI Kids study protocol. *BMJ Open*. 2019;9(4):e025897.
22. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009;6(5):359–62.
23. Milacic M, Beavers D, Conley P, Gong C, Gillespie M, Griss J, et al. The Reactome Pathway Knowledgebase 2024. *Nucleic Acids Res*. 2023;52(D1):D672–8.
24. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene Ontology: tool for the unification of biology. *Nat Genet*. 2000;25(1):25–9.
25. Consortium TGO, Aleksander SA, Balhoff J, Carbon S, Cherry JM, Drabkin HJ et al. The Gene Ontology knowledgebase in 2023. *Genetics*. 2023;224(1).
26. Perez-Riverol Y, Bandla C, Kundu Deepti J, Kamatchinathan S, Bai J, Hewapathirana S, et al. The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res*. 2025;53(D1):D543–53.
27. Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, Hachilif R, et al. The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res*. 2023;51(D1):D638–46.
28. Dunstan-Harrison C, Morison IM, Ledgerwood EC. A novel frameshift GP1BB mutation causes autosomal dominant macrothrombocytopenia with decreased vWF receptor expression but normal platelet aggregation. *Platelets*. 2022;33(2):324–7.

29. Wang C-z, Zhang Z-q, Zhang Y, Zheng L-f, Liu Y, Yan A-t, et al. Comprehensive characterization of TGF β 1 across hematological malignancies. *Sci Rep*. 2023;13(1):19107.
30. Blank U, Karlsson S. TGF- β signaling in the control of hematopoietic stem cells. *Blood*. 2015;125(23):3542–50.
31. Gostynska S, Venkatesan T, Subramani K, Cortez B, Robertson A, Subrahmanian S, et al. Megakaryocyte/platelet-derived TGF- β 1 inhibits megakaryopoiesis in bone marrow by regulating thrombopoietin production in liver. *Blood Adv*. 2022;6(11):3321–8.
32. Ishizaki J, Suzuki N, Higashino K, Yokota Y, Ono T, Kawamoto K, et al. Cloning and characterization of novel mouse and human secretory phospholipase A(2)s. *J Biol Chem*. 1999;274(35):24973–9.
33. Suzuki N, Ishizaki J, Yokota Y, Higashino K, Ono T, Ikeda M, et al. Structures, enzymatic properties, and expression of novel human and mouse secretory phospholipase A(2)s. *J Biol Chem*. 2000;275(8):5785–93.
34. Kramer RM, Hession C, Johansen B, Hayes G, McGray P, Chow EP, et al. Structure and properties of a human non-pancreatic phospholipase A2. *J Biol Chem*. 1989;264(10):5768–75.
35. Dahlbäck B, Villoutreix BO. Regulation of Blood Coagulation by the Protein C Anticoagulant Pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2005;25(7):1311–20.
36. Boni LT, Rando RR. The nature of protein kinase C activation by physically defined phospholipid vesicles and diacylglycerols. *J Biol Chem*. 1985;260(19):10819–25.
37. Freyssinet JM, Gauchy J, Cazenave JP. The effect of phospholipids on the activation of protein C by the human thrombin-thrombomodulin complex. *Biochem J*. 1986;238(1):151–7.
38. Fujita M, Zhu K, Fujita CK, Zhao M, Lam KS, Kurth MJ, et al. Proinflammatory secreted phospholipase A2 type IIA (sPLA-IIA) induces integrin activation through direct binding to a newly identified binding site (site 2) in integrins $\alpha v\beta 3$, $\alpha 4\beta 1$, and $\alpha 5\beta 1$. *J Biol Chem*. 2015;290(1):259–71.
39. Saegusa J, Akakura N, Wu CY, Hoogland C, Ma Z, Lam KS, et al. Pro-inflammatory secretory phospholipase A2 type IIA binds to integrins $\alpha v\beta 3$ and $\alpha 4\beta 1$ and induces proliferation of monocytic cells in an integrin-dependent manner. *J Biol Chem*. 2008;283(38):26107–15.
40. Shinton SA, Brill-Dashoff J, Hayakawa K. Pla2g2a promotes innate Th2-type immunity lymphocytes to increase B1a cells. *Sci Rep*. 2022;12(1):14899.
41. Hertz MI, Landry DM, Willis AE, Luo G, Thompson SR. Ribosomal protein S25 dependency reveals a common mechanism for diverse internal ribosome entry sites and ribosome shunting. *Mol Cell Biol*. 2013;33(5):1016–26.
42. Das AS, Basu A, Mukhopadhyay R. Ribosomal proteins: the missing piece in the inflammation puzzle? *Mol Cell Biochem*. 2025;480(2):785–97.
43. Zhou X, Liao W-J, Liao J-M, Liao P, Lu H. Ribosomal proteins: functions beyond the ribosome. *J Mol Cell Biol*. 2015;7(2):92–104.
44. Zhang X, Wang W, Wang H, Wang M, Xu W, Zhang R. Identification of ribosomal protein S25 (RPS25)–MDM2–p53 regulatory feedback loop. *Oncogene*. 2013;32(22):2782–91.
45. Nukala SB, Regazzoni L, Aldini G, Zodda E, Tura-Ceide O, Mills NL, et al. Differentially Expressed Proteins in Primary Endothelial Cells Derived From Patients With Acute Myocardial Infarction. *Hypertension*. 2019;74(4):947–56.
46. Choudry FA. Novel insights into megakaryopoiesis, thrombopoiesis and acute coronary thrombosis: transcriptome profiling of the haematopoietic stem cell, megakaryocyte and platelet. 2018.
47. Visscher MO, Hu P, Carr AN, Bascom CC, Isfort RJ, Creswell K, et al. Newborn infant skin gene expression: Remarkable differences versus adults. *PLoS ONE*. 2021;16(10):e0258554.
48. Cagnin S, Pontisso P, Martini A. SerpinB3: A Multifaceted Player in Health and Disease—Review and Future Perspectives. *Cancers*. 2024;16(14):2579.
49. Wang S, Luke CJ, Pak SC, Shi V, Chen L, Moore J, et al. SERPINB3 (SCCA1) inhibits cathepsin L and lysoptosis, protecting cervical cancer cells from chemoradiation. *Commun Biology*. 2022;5(1):46.
50. Wu J, Zhang K, Liao Y, Yang C, Ge J, Wu C, et al. Serum SERPINB3/4 in Moderate-to-Severe Prurigo Nodularis: A Potential Biomarker for Disease Severity and Eosinophil-Related Parameters. *Immun Inflamm Dis*. 2025;13(9):e70263.
51. Turato C, Vitale A, Fasolato S, Ruvoletto M, Terrin L, Quarta S, et al. SERPINB3 is associated with TGF- β 1 and cytoplasmic β -catenin expression in hepatocellular carcinomas with poor prognosis. *Br J Cancer*. 2014;110(11):2708–15.
52. Fadini GP, Albiero M, Million R, Poncina N, Rigato M, Scotton R, et al. The molecular signature of impaired diabetic wound healing identifies serpinB3 as a healing biomarker. *Diabetologia*. 2014;57(9):1947–56.
53. Lauko A, Turaga S, Volovetz J, Bayik D, Naik U, Lathia J. STEM-07. IDENTIFICATION OF A NOVEL CSC SIGNALING AXIS CONTAINING JAM-A/SERPINB3/TGF-BETA. *Neurooncology*. 2020;22(Supplement2):ii197–8.
54. Turato C, Calabrese F, Biasiolo A, Quarta S, Ruvoletto M, Tono N, et al. SERPINB3 modulates TGF- β expression in chronic liver disease. *Lab Invest*. 2010;90(7):1016–23.
55. Thapa N, Lee B-H, Kim I-S. TGF β 1/ β ig-h3 protein: A versatile matrix molecule induced by TGF- β . *Int J Biochem Cell Biol*. 2007;39(12):2183–94.
56. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. *BMC Bioinformatics*. 2009;10:421.
57. Nishioka J, Ning M, Hayashi T, Suzuki K. Protein C inhibitor secreted from activated platelets efficiently inhibits activated protein C on phosphatidylethanolamine of platelet membrane and microvesicles. *J Biol Chem*. 1998;273(18):11281–7.
58. Elisen MG, von dem Borne PA, Bouma BN, Meijers JC. Protein C inhibitor acts as a procoagulant by inhibiting the thrombomodulin-induced activation of protein C in human plasma. *Blood*. 1998;91(5):1542–7.
59. Cooper ST, Whinna HC, Jackson TP, Boyd JM, Church FC. Intermolecular interactions between protein C inhibitor and coagulation proteases. *Biochemistry*. 1995;34(40):12991–7.

60. Meijers JC, Kanter DH, Vlooswijk RA, van Erp HE, Hessing M, Bouma BN. Inactivation of human plasma kallikrein and factor XIa by protein C inhibitor. *Biochemistry*. 1988;27(12):4231–7.
61. Boldt ABW, Boschmann SE, Catarino SJ, Andrade FA, de Messias-Reason IJ. MASP1 and MASP2. In: Choi S, editor. *Encyclopedia of Signaling Molecules*. New York, NY: Springer New York; 2017. pp. 1–18.
62. Garlatti V, Belloy N, Martin L, Lacroix M, Matsushita M, Endo Y, et al. Structural insights into the innate immune recognition specificities of L- and H-ficolins. *Embo j*. 2007;26(2):623–33.
63. Krott KJ, Feige T, Bosbach A, Beele AN, Krüger I, Reusswig F, et al. Interplay Between Thrombospondin-1 and CD36 Modulates Platelet-RBC Interaction in Thrombosis and Abdominal Aneurysm Formation. *Circ Res*. 2025;137(12):1459–79.
64. Sanjurjo L, Amézaga N, Aran G, Naranjo-Gómez M, Arias L, Armengol C, et al. The human CD5L/AIM-CD36 axis: A novel autophagy inducer in macrophages that modulates inflammatory responses. *Autophagy*. 2015;11(3):487–502.
65. Sanchez-Moral L, Ráfols N, Martori C, Paul T, Téllez É, Sarrias MR. Multifaceted Roles of CD5L in Infectious and Sterile Inflammation. *Int J Mol Sci*. 2021;22(8).
66. Amézaga N, Sanjurjo L, Julve J, Aran G, Pérez-Cabezas B, Bastos-Amador P, et al. Human scavenger protein AIM increases foam cell formation and CD36-mediated oxLDL uptake. *J Leukoc Biol*. 2014;95(3):509–20.
67. Sanjurjo L, Aran G, Roher N, Valledor AF, Sarrias MR. AIM/CD5L: a key protein in the control of immune homeostasis and inflammatory disease. *J Leukoc Biol*. 2015;98(2):173–84.
68. Wang C, Yosef N, Gaublomme J, Wu C, Lee Y, Clish CB, et al. CD5L/AIM Regulates Lipid Biosynthesis and Restrains Th17 Cell Pathogenicity. *Cell*. 2015;163(6):1413–27.
69. Sarrias MR, Roselló S, Sánchez-Barbero F, Sierra JM, Vila J, Yélamos J, et al. A role for human Sp alpha as a pattern recognition receptor. *J Biol Chem*. 2005;280(42):35391–8.
70. Murray SS, Wang JC, Duarte ME, Zhao KW, Tian H, Francis T, et al. The bone matrix protein secreted phosphoprotein 24 kD (Spp24): bone metabolism regulator and starting material for biotherapeutic materials. *Histol Histopathol*. 2015;30(5):531–7.
71. Yuste-Checa P, Carvajal AI, Mi C, Paatz S, Hartl FU, Bracher A. Structural analyses define the molecular basis of clusterin chaperone function. *Nat Struct Mol Biol*. 2025;32(10):2035–45.
72. Rodríguez-Rivera C, Garcia MM, Molina-Álvarez M, González-Martín C, Goicoechea C, Clusterin. Always protecting. Synthesis, function and potential issues. *Biomed Pharmacother*. 2021;134:111174.

Figures

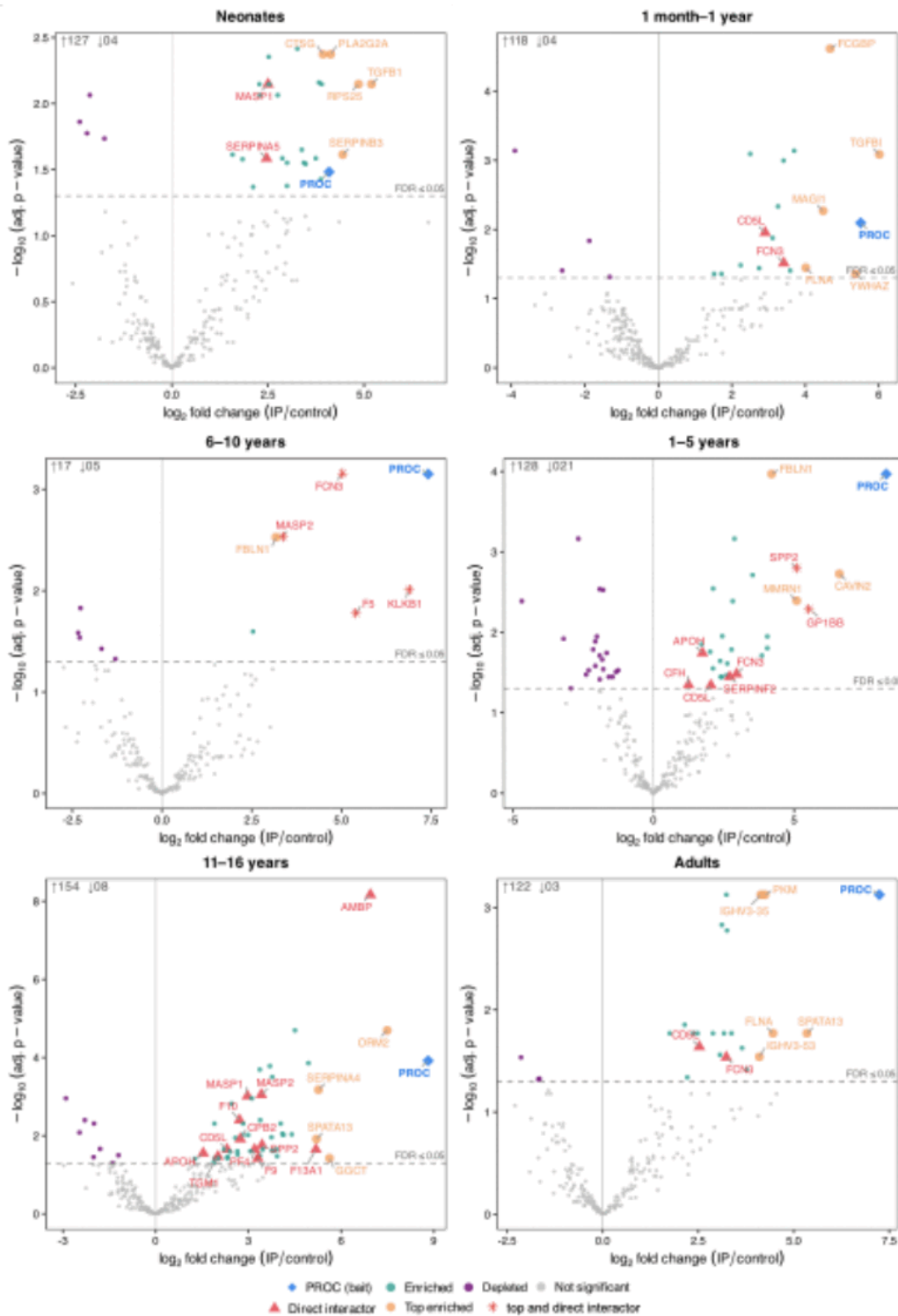


Figure 1

Differential protein enrichment and depletion between IP and controls for each age group. Protein C (PROC) as bait protein is indicated in blue, most highly enriched are highlighted in yellow, and proteins predicted by STRING analysis as direct interactors are highlighted in red. Limma (v.3.52.4) was used to perform pair-wise comparisons. A protein was determined to be significantly differentially expressed if the false discovery rate (FDR) was ≤ 0.05 .

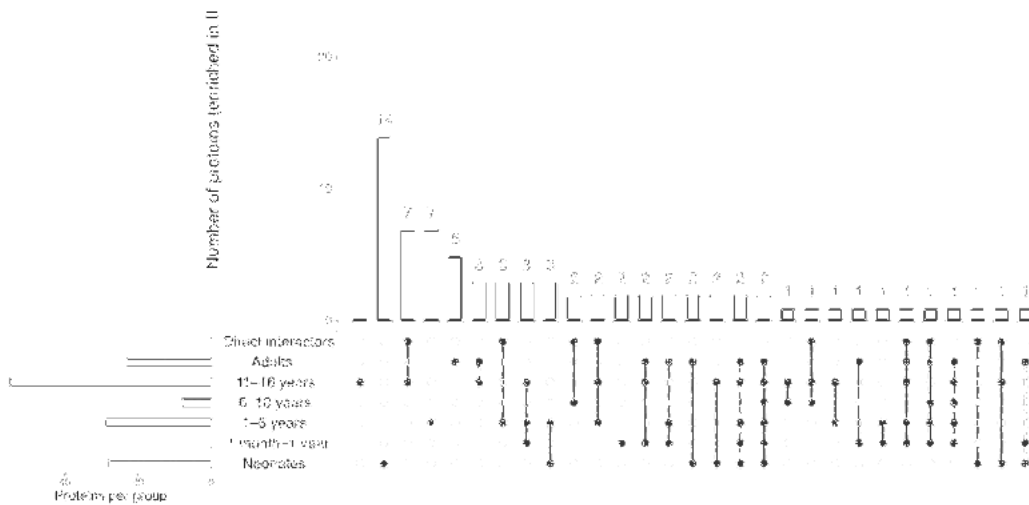


Figure 2

Unique and shared enriched proteins. Upset plot depicting the number of unique and shared statistically significant proteins that have been enriched in the Protein C CoIP within each age group and the number of proteins predicted to be direct interactors of Protein C by STRING analysis.

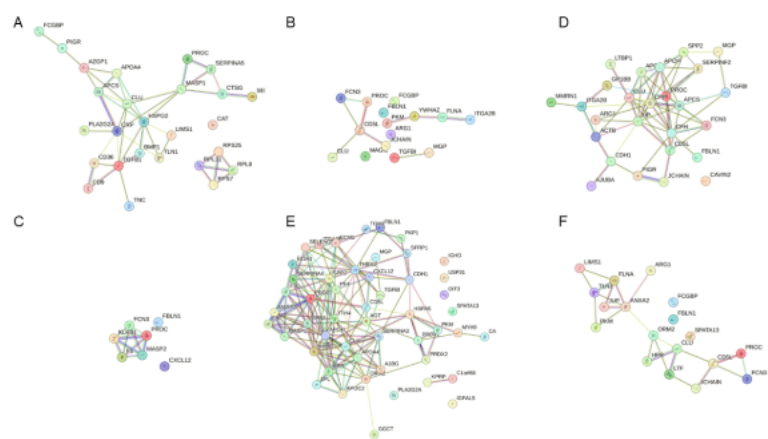


Figure 3

Protein network analysis of the full list of significantly enriched proteins from each age group. Analysis performed using STRING v12.0, showing full STRING network with all interaction sources, a minimum required interaction score of 0.4 and no clustering (A) Neonates. (B) 1 month – 1 year. (C) 1 – 5 years. (D) 6 – 10 years. (E) 11 – 16 years. (F) Adult. Line thickness indicates strength of data support. Bait protein of Protein C (PROC) indicated in red.



Figure 4

GO biological process (GO-BP) pathways enriched in CoIPs for each age group. Enriched GO terms showing the top 15 significant enriched terms per age group. (A) Enriched processes whose gene list may or may not include PROC. (B) Enriched processes whose gene list specifically includes PROC.

Supplementary Files

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