

Percília Victória Santos de Oliveira

Proteína dissulfeto isomerase plasmática: detecção e correlação com assinaturas proteômicas ligadas a distintos fenótipos endoteliais em indivíduos saudáveis

Tese apresentada a Faculdade de Medicina da
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Programa de Cardiologia

Orientador: Prof. Dr. Francisco Rafael Martins
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Dados Internacionais de Catalogação na Publicação (CIP)

Preparada pela Biblioteca da
Faculdade de Medicina da Universidade de São Paulo

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Oliveira, Percillia Victória Santos de
Proteína dissulfeto isomerase plasmática :
detecção e correlação com assinaturas proteômicas
ligadas a distintos fenótipos endoteliais em
indivíduos saudáveis / Percillia Victória Santos de
Oliveira. -- São Paulo, 2019.

Tese (doutorado) -- Faculdade de Medicina da
Universidade de São Paulo.

Programa de Cardiologia.

Orientador: Francisco Rafael Martins Laurindo.

Descritores: 1. Isomerases de dissulfetos de
proteínas 2. Plasma 3. Proteômica 4. Proteínas sanguíneas
5. Células endoteliais 6. Espaço extracelular

USP/FM/DBD-072/19

Responsável: Erinalva da Conceição Batista, CRB-8 6755

Dedico este trabalho aos meus pais.

A vocês que me apresentaram o caminho da honestidade e persistência; também pelo apoio, incentivo e amor.

Agradecimentos

Ao Prof. Francisco Laurindo pela orientação e mentoria durante o desenvolvimento deste estudo. Pelo exemplo de cientista e constante apoio que foram além da supervisão do doutorado; considero um aprendizado diário de como fazer ciência, encontrar questionamentos relevantes, escrever cientificamente e buscar conhecimento. Agradeço pela paciência, gentileza e disponibilidade de sempre. Muito obrigada por todas as oportunidades e confiança depositas em mim e no meu trabalho; por me permitir aprender as lições profissionais que a jornada do doutorado me proporcionou, que foram essenciais para o meu amadurecimento científico.

Aos colaboradores: Prof. Marcelo Santoro pela contribuição científica, discussões, críticas e sugestões valiosas. Ana Sachetto pela ajuda com os ensaios de agregação plaquetária e também pelas discussões e cafés. Ao Prof. Daniel Martins-de-Souza e Sheila Garcia-Rosa pela colaboração imprescindível com análise proteômica e discussão de resultados. Ao Prof. Alexandre Pereira, pela disponibilização do banco de plasmas utilizado neste estudo e discussões.

Aos voluntários que participaram do estudo e estiveram disponíveis durante todo o período de execução do trabalho.

À Patrícia minha amiga e companheira de aventuras na vida e no doutorado. A aventura em São Paulo começou juntas e de lá para cá passamos por poucas e boas. Tantas histórias de felicidade e desespero, mas sem “nós - as baianas”, não sei se teria conseguido superar o nosso início aqui. Por isso, deixo aqui o meu agradecimento especial.

Às amigas de Laboratório e de vida Carolina e Tiffany agradeço pela amizade e companheirismo. Obrigada pela ajuda... Carol nas coletas de sangue e Tiffany com a migração celular; além de todos os momentos compartilhados fora do lab. Vocês são gratas surpresas de São Paulo, e fico feliz em tê-las por perto. Muito obrigada!

Ao Victor sou grata por toda a parceria dentro e fora do laboratório. Obrigada pela ajuda em vários momentos na bancada e auxílio na organização do laboratório como um todo. Obrigada pela amizade.

À Thais e sua família por terem me acolhido em São Paulo, me recebido em sua casa e compartilhado um pouco da vida fora do laboratório no início da minha jornada aqui. Agradeço pela co-orientação durante meu período de estágio no laboratório e por tudo que aprendi durante esse tempo de colaboração.

À Thayna por ter sido a responsável pela minha indicação e contato inicial para estágio no laboratório.

A todos do Laboratório de Biologia Vascular: Ana Moretti pela colaboração neste trabalho e por todas as discussões científicas e não científicas durante esses anos. Obrigada por estar sempre disposta a me ajudar; Laura pela amizade e ajuda nas coletas de sangue. Sou grata pelo seu carinho comigo; Leonardo pelas discussões científicas e momentos compartilhados dentro e fora do laboratório; Fernando, Ana Cecília, João, Maria, Arruda, Leonora, Denise, Daniela e Luciana pelos momentos compartilhados e ajuda em algum momento; Jéssyca (ex-LBV), pela amizade e convivência no dia-a-dia durante nossa época juntas no doutorado.

À Nathália pela colaboração e dedicação no desenvolvimento do projeto de iniciação científica e coleguismo construído.

Aos amigos de bandeirão e pós-graduação Darlan, Amanda e Fernanda. Obrigada pelos momentos compartilhados.

Aos outros amigos que tornaram esses anos em São Paulo mais fáceis de serem vividos... Samir, Jamile, Priscila agradeço pela amizade e por todas as nossas aventuras.

A meus pais, João e Sileildes, a quem sou mais grata. Por terem me apoiado a vir para São Paulo no momento mais difícil de nossas vidas. Obrigada por não medirem esforços pela minha formação, pelo apoio, incentivo e amor. Amo vocês!

À minha Vózinha que sempre esteve presente mesmo estando longe e sempre faz questão de me ajudar. Te amo!

A todos familiares que sempre torceram por mim. Me perdoem as ausências. Tias (Fátima, Ediléia, Creusa e família) muito obrigada pelo apoio e orações. Aos meus amigos: Kátia, Saulo, Bruna, Camila, Lisiane, Wanessa, obrigada pela amizade e por estarem presentes mesmo distantes.

A Deus por ser minha fortaleza e me permitir o fazer no empenho que me cabe.

Este trabalho foi financiado pela FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo); Processo 2014/20595-1 (bolsa de doutorado direto); Projeto FAPESP 2013/07937-8 (CEPID de Processos Redox em Biomedicina – Redoxoma).

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Universidade de São Paulo. Faculdade de Medicina. Divisão de Biblioteca e Documentação. *Guia de apresentação de dissertações, teses e monografias*. Elaborado por Anneliese Carneiro da Cunha, Maria Julia de A. L. Freddi, Maria F. Crestana, Marinalva de Souza Aragão, Suely Campos Cardoso, Valéria Vilhena. 3a ed. São Paulo: Divisão de Biblioteca e Documentação; 2011.

Abreviaturas dos títulos dos periódicos de acordo com *List of Journals Indexed in Index Medicus*.

Sumário

Lista de abreviaturas

Lista de símbolos

Resumo

Summary

1. Introdução.....	1
1.1. Proteína Dissulfeto Isomerase: estrutura e função no retículo endoplasmático	4
1.2. PDI fora do retículo endoplasmático: PDI epi/pericelular (<i>pecPDI</i>).....	8
2. Objetivos	12
2.1. Objetivo geral	13
2.2. Objetivos específicos.....	13
3. Métodos.....	14
3.1. Reagentes.....	15
3.2. População de estudo	15
3.3. ELISA (Enzyme-Linked Immunosorbent Assay)	16
3.4. Expressão e purificação da PDI recombinante	16
3.5. Preparo da PDI reduzida, oxidada e S-nitrosilada.....	17
3.6. Expressão proteica por western blot.....	17
3.7. Obtenção de plasma pobre em plaquetas.....	18
3.8. Imunoprecipitação	18
3.9. Ensaio de atividade tiol-redutase da PDI no plasma	18
3.10. Separação e lise de micropartículas plasmáticas	20
3.11. Ensaio para detecção do estado redox da PDI no plasma utilizando sondas biotiniladas	21
3.12. Cultura de células.....	22
3.13. Incubação de células endoteliais com plasma.....	22
3.14. Ensaio de viabilidade celular	22
3.15. Análise proteômica	23
3.15.1. Preparo de amostra e análise UPLC-HDMSE	23
3.15.1.1. Amostras de plasma	23

3.15.1.2. Meio condicionado (MC) de HUVEC	23
3.15.2. Método de aquisição (espectrometria de massa)	24
3.15.3. Identificação e quantificação de proteínas	24
3.15.4. Análise de enriquecimento de vias	25
3.16. Agregação plaquetária	25
3.17. Expressão de RNA mensageiro por PCR em tempo real.....	26
3.18. Ensaio de adesão celular	26
3.19. Ensaio de migração celular (<i>Wound healing</i>)	27
3.20. Análise estatística	27
4. Resultados	29
4.1. Validação do ensaio de ELISA para detecção da PDI.....	30
4.2. Concentração plasmática da PDI mostra alta variabilidade interindividual, porém baixa variabilidade intraindividual	35
4.3. Estado redox do pool de PDI no plasma.....	40
4.4. Plasmas com baixas vs. altas concentrações de PDI revelam distintos perfis de composição de proteínas.....	42
4.5. Perfis proteômicos plasmáticos discriminados pela PDI não se correlacionaram com padrão de agregação plaquetária.....	45
4.6. Diferentes concentrações de PDI no plasma recapitularam perfis opostos de expressão gênica e secreção em células endoteliais	50
4.7. Correlação entre concentrações de PDI e função de células endoteliais	60
5. Discussão.....	66
6. Conclusões	74
7. Anexos	77
8.Referências	96
9. Apêndice.....	107

Lista de abreviaturas

ADP	Adenosina difosfato
AVC	Acidente Vascular Cerebral
Bep1a	Bepristat 1a
Bep2a	Bepristat 2a
cDNA	DNA complementar
CE	Células endoteliais
CV	Coefficiente de Variação
DEMEM	Dulbecco's Modified Eagle's Medium
DO	Densidade óptica
DTT	1,4-Ditiotreitol
ECV	Evento cardiovascular
EDTA	Ácido Etilenodiamino Tetra-acético
ELISA	Ensaio de Imunoabsorção Enzimática
ERAD	Via de degradação associada ao RE
Ero1	Oxidoreductina do Retículo Endoplasmático
ERp5	Proteína do retículo endoplasmático 5 (PDIA6)
ERp57	Proteína do retículo endoplasmático 57 (PDIA3)
ERp72	Proteína do retículo endoplasmático 72 (PDIA4)
FBS	Soro fetal bovino

GO	Ontologia Gênica
GSH	Glutathiona reduzida
GSSG	Glutathiona oxidada
HCT116	Linhagem celular de Carcinoma de Cólon Retal Humano
HDL	Lipoproteína de alta densidade
HF	Hipercolesterolemia Familiar
HKE3	Linhagem Celular de Carcinoma de Cólon Retal Humano Mutada
HRG	Glicoproteína rica em histidina
HSP	Proteínas de choque térmico
IAM	Infarto Agudo do Miocárdio
IMC	Índice de massa corpórea
LDL	Lipoproteína de Baixa Densidade
MARS-14	Coluna de Múltipla Afinidade Removedora de 14 Proteínas
MC	Meio condicionado
MP	Micropartículas
MPB	3-(N-maleimido-propionyl) biocytin
mRNA	RNA mensageiro
PBS	Tampão Fosfato Salino
PCR	Reação em Cadeia da Polimerase
PDI	Proteína Dissulfeto Isomerase
pecPDI	Proteína Dissulfeto Isomerase Peri/epicelular

PP-PDI	Plasma pobre em PDI
PRP	Plasma rico em Plaquetas
PR-PDI	Plasma rico em PDI
RE	Retículo endoplasmático
RPMI	Roswell Park Memorial Institute medium
TA	Temperatura ambiente
TB	<i>Terrific Broth</i>
TBS-T	Tampão Salino Tris Tween 20
TMB	Tetrametilbenzidina
Vs.	“Versus”

Lista de símbolos

μL	Microlitro
μm	Micrometro
dL	Decilitro
h	Hora
g	Força gravitacional
kg	Quilograma
L	Litro
M	Molar
mg	Miligrama
min	Minuto
mL	Mililitro
mM	Milimolar
ng	Nanograma
nm	Nanômetro
pg	Picograma
RFU	Unidade relativa de fluorescência
rpm	Rotações por minuto

Lista de figuras

Figura 1. Painel de reatividade de tióis.....	3
Figura 2. Proteína Dissulfeto Isomerase.....	7
Figura 3 – Representação da reação tiol redutase da PDI.	19
Figura 4 – Detecção da PDI recombinante pelo kit de ELISA ‘ <i>Human P4HB Par Set</i>	31
Figura 5 – Validação do ensaio de ELISA para deteção específica da PDI.	32
Figura 6 – PDI plasmática detectada por imunoprecipitação.	33
Figura 7 – Atividade redutase da PDI no plasma.	34
Figura 8 – Concentração plasmática da PDI em indivíduos saudáveis.	36
Figura 9 – Características da PDI no plasma.....	38
Figura 10 – Correlação entre a concentração plasmática da PDI e diferentes variáveis individuais.	39
Figura 11 – Detecção do estado redox da PDI no plasma humano.	41
Figura 12 – Proteoma total e análise de enriquecimento de vias e processos biológicos de proteínas diferencialmente expressas nos PP-PDI e PR-PDI.	44
Figura 13 – Processos de ontologia genica (<i>gene ontology, GO</i>) das proteínas identificadas no proteoma total do plasma.....	45
Figura 14 – Agregação plaquetária em plasma rico em plaquetas.	47
Figura 15 – Agregação plaquetária em sangue total.....	48
Figura 16 – Detecção da PDI solúvel nos plasmas após agregação plaquetária.	49
Figura 17 – Viabilidade de células endoteliais (HUVEC) incubadas com PP-PDI e PR-PDI.	51
Figura 18 – <i>Heat map</i> do mRNA de genes envolvidos em diversos eventos celulares após a exposição de células endoteliais a PP-PDI e PR-PDI.	53

Figura 19 – Expressão do mRNA para os genes que tiveram expressão simultaneamente aumentada nas células expostas aos PP- PDI e diminuída naquelas expostas aos PR-PDI...	54
Figura 20 – Expressão do mRNA para os genes que tiveram comportamentos distintos nas células endoteliais expostas aos PP-PDI ou PR-PDI.....	55
Figura 21 – Proteoma e análise de enriquecimento de vias e processos biológicos de proteínas diferencialmente expressas no secretoma de células endoteliais expostas aos PP-PDI ou PR-PDI.	57
22 – Processos de ontologia genica (<i>gene ontology, GO</i>) das proteínas identificadas no secretoma de células endoteliais expostas aos PP-PDI ou PR-PDI.....	58
23 – Expressão da PDI e outros membros da família (ERp57, ERp72) no secretoma de células endoteliais.....	59
Figura 24 – Efeito da incubação dos PP-PDI e PR-PDI na adesão de células endoteliais à fibronectina.....	61
Figura 25 – Efeito da incubação dos PP-PDI e PR-PDI na adesão de células endoteliais ao colágeno do tipo-I.....	62
Figura 26 – Efeito da incubação dos PP-PDI e PR-PDI na migração de células endoteliais.....	64
Figura 27 – Concentração circulante da PDI em pacientes do biobanco e controles saudáveis.....	72
Figura 28 – Resumo gráfico.	74

Lista de tabelas

Tabela 1. Característica da população de indivíduos saudáveis.	35
Tabela 2. Características clínicas da população de pacientes do biobanco.	65

Resumo

Oliveira PVS. *Proteína dissulfeto isomerase plasmática: detecção e correlação com assinaturas proteômicas ligadas a distintos fenótipos endoteliais em indivíduos saudáveis* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2019.

A Dissulfeto Isomerase Proteica (PDI) é uma chaperona ditiol-dissulfeto oxidoreductase da superfamília tiorredoxina que catalisa o enovelamento de proteínas secretadas ou de membrana por meio da introdução, redução ou isomerização de pontes dissulfeto. A PDI é primariamente localizada no lúmen do retículo endoplasmático, no entanto a presença de uma pequena fração da PDI na superfície celular e no meio extracelular tem sido documentada em diversos tipos celulares. Particularmente em plaquetas e células endoteliais, a PDI epi/pericelular (pecPDI) está envolvida em diversos processos incluindo ativação de plaquetas/trombose, infecções virais e remodelamento vascular. A ausência de PDI (e outras tiol isomerases) na circulação tem sido proposta como mecanismo para prevenir trombose na ausência de lesão vascular. No entanto, esta questão permanece obscura e existe pouca informação sobre a concentração circulante da PDI e outras tiol isomerases vasculares. Neste estudo, investigamos a ocorrência e implicações fisiológicas de um *pool* circulante de PDI em indivíduos saudáveis e validamos um ensaio para detecção da PDI. Os resultados mostraram um *pool* detectável de PDI no plasma por ELISA, confirmados por imunoprecipitação e ensaio de atividade (inibição da redução da sonda di-eosina-GSSG pela rutina, um inibidor específico da PDI). A concentração de PDI no plasma (mediana=330 pg/mL) indicam uma alta variabilidade interindividual, com valores muito baixos/indetectáveis (plasmas pobres em PDI [PP-PDI], definidos como \leq 330 pg/mL) até valores superiores a 1000 pg/mL (plasmas ricos em PDI [PR-PDI], designados como $>$ 330 pg/mL). Por outro lado, um resultado importante foi o fato de que valores de PDI mostraram variabilidade intraindividual muito baixa ao longo do tempo, detectada através de medidas repetidas em diferentes ocasiões e/ou condições. A fração de PDI presente em micropartículas plasmáticas foi variável, mas em geral pequena em relação ao *pool* total de PDI. O *pool* da PDI no plasma está majoritariamente reduzido (60–80%) sem diferenças entre os grupos PP-PDI e PR-PDI. Importante, os valores de PDI associaram-se a distintos perfis proteômicos plasmáticos. Enquanto os PR-PDI se

associaram preferencialmente a proteínas relacionadas a diferenciação celular, processamento de proteínas, funções *housekeepings*, entre outras, os PP-PDI mostraram expressão diferencial de proteínas associadas a coagulação, respostas inflamatórias e imunoativação. A atividade de plaquetas medida por agregação foi semelhante entre os indivíduos com PP-PDI vs. PR-PDI. No entanto, a PDI solúvel foi diminuída após agregação plaquetária na maioria dos indivíduos em ambos os grupos, sugerindo captura devida a exposição de moléculas adesivas. Em outras séries de experimentos, mostramos que tais perfis proteômicos plasmáticos se correlacionaram ao fenótipo e função endotelial. Células endoteliais em cultura incubadas com PP-PDI ou PR-PDI recapitularam padrões de expressão gênica e de secreção de proteínas similares aos perfis plasmáticos correspondentes. Além disso, as assinaturas proteômicas identificadas em ambos os tipos de plasma traduziram-se em distintas respostas funcionais endoteliais. Os PP-PDI promoveram comprometimento da adesão de células endoteliais à fibronectina e perturbaram o padrão de migração celular associado à reparação de lesão endotelial. Em contraste, os PR-PDI não afetaram significativamente a adesão celular e sustentaram um padrão de migração organizado. Em outra população de pacientes com eventos cardiovasculares, os valores de PDI no plasma (mediana= 35 pg/mL) foram significativamente inferiores aos de indivíduos saudáveis. Em conclusão, o *pool* detectável de PDI presente no plasma se associou a distintos perfis proteômicos e parece se comportar como um indicador/ marcador de assinaturas proteômicas relacionadas à função e sinalização endotelial. Este é o primeiro estudo descrevendo valores circulantes de PDI diretamente relacionados a distintos fenótipos endoteliais.

Descritores: isomerases de dissulfetos de proteínas; plasma; proteômica; proteínas sanguíneas; células endoteliais; espaço extracelular.

Summary

Oliveira PVS. *Protein disulfide isomerase plasma levels in healthy humans reveal proteomic signatures involved in contrasting endothelial phenotypes* [thesis]. São Paulo: “Faculdade de Medicina, Universidade de São Paulo”; 2019.

Protein disulfide isomerase (PDI) is a dithiol–disulfide oxidoreductase chaperone from thioredoxin superfamily which catalyzes introduction, reduction or isomerization of disulfide bonds in nascent proteins, typically destined to extracellular secretion or membrane insertion. PDI is primarily located into the endoplasmic reticulum; however, there are clear evidences for the presence of a small PDI fraction at the cell surface and extracellular milieu in several cell types. Particularly in platelets and endothelial cells, such peri/epicellular pool of PDIA1 (*pecPDI*) is involved in distinct processes including platelet activation/thrombosis, viral infection and vascular remodeling. The absence of PDI (and other thiol isomerases) from circulating plasma has been proposed as a mechanism to prevent thrombogenesis in the absence of vascular injury. However, this question remains unclear, as there is little information on the circulating levels of PDI and other vascular thiol isomerases. Here we investigated the occurrence and physiological significance of a circulating pool of PDI in healthy humans. We validated an assay for detecting PDI in plasma of healthy individuals. The results showed a detectable pool of plasma PDI by ELISA, confirmed by immunoprecipitation and activity assay (diesoin-GSSG inhibitable by rutin, a specific PDI inhibitor). PDI levels (median= 330 pg/mL) exhibited high inter-individual variability, ranging from undetectable/low (PDI-poor plasma, defined as ≤ 330 pg/mL) until 1000 pg/mL (PDI-rich plasma, >330 pg/mL). Remarkably, opposite to inter-individual variability, the intra-individual variability was quite low, so that values assessed under distinct conditions over time were close and reproducible. The majority (60-80%) of plasma PDI is in the reduced state, without any difference among individuals with PDI-poor and PDI-rich plasma. Importantly, plasma PDI levels could discriminate between distinct plasma proteome signatures, with PDI-rich plasma differentially expressing proteins related to cell differentiation, protein processing, housekeeping functions and others, while PDI-poor plasma differentially displayed proteins associated with coagulation, inflammatory responses and immunoactivation. Platelet activity assessed by

aggregation was similar between PDI-poor vs. PDI-rich plasma. However, soluble PDI was decreased after platelet activation in both groups, suggesting sequestration of platelet-derived PDI by its potential substrates. In other set of experiments, we showed that such protein signatures closely correlated with endothelial function and phenotype, since cultured endothelial cells incubated with PDI-poor or PDI-rich plasma recapitulated gene expression and secretome patterns in line with their corresponding plasma signatures. Furthermore, such signatures translated into functional responses, with PDI-poor plasma promoting impairment of endothelial adhesion to fibronectin and a disturbed pattern of wound-associated migration and recovery area. In contrast, PDI-rich plasma did not significantly affect cell adhesion and supported organized endothelial migration. In another dataset, patients with cardiovascular events had lower PDI levels (median= 35 pg/mL) vs. healthy individuals. In conclusion, a PDI pool detectable in plasma from healthy individuals is associated with distinct proteomic profiles and seems to behave as an indicator/marker of proteomic signatures related with endothelial function and signaling. This is the first study describing PDI levels as reporters of specific plasma proteome signatures directly promoting contrasting endothelial phenotypes and functional responses.

Descriptors: protein disulfide-isomerases; plasma; proteomics; blood proteins; endothelial cells; extracellular space.

1. Introdução

Um importante avanço no campo da biologia redox foi o entendimento do papel de reações de transferência de elétrons envolvendo intermediários radicalares ou não-radicalares em vias de sinalização celular, a chamada *sinalização redox* (1). O conceito de sinalização redox pode ser visto como uma revolução na biomedicina redox, tendo transferido o foco de processos tóxicos e lesão biomolecular para uma visão mais abrangente de estresse oxidativo (2). Neste contexto, estresse oxidativo representa o desequilíbrio, ou em uma abordagem sistemática a perda da modularidade, da sinalização redox celular (3). Tais mudanças trazem importantes implicações, muito além de uma discussão conceitual. Do ponto de vista da biomedicina redox, aprimoram a compreensão dos importantes desafios representados pelas tentativas de modulação do estresse oxidativo em doenças e do encontro de biomarcadores pertinentes, algo extremamente investigado na literatura da área, mas com resultados efetivos ainda incipientes (4). Neste sentido, a biomedicina redox tem cada vez mais se estabelecido como uma disciplina independente, em boa parte devido à enorme e crescente complexidade.

Em paralelo, do ponto de vista mecanístico, há também importantes desafios para o entendimento de como intermediários oxidantes, por exemplo peróxido de hidrogênio, podem exercer sinalização celular em alvos específicos. Os principais grupos redox-sensíveis em proteínas são metais redox (que não serão discutidos neste trabalho) e grupos tiol proteicos, associados majoritariamente a cisteínas redox. Estes têm a capacidade de mudar seu estado redox de uma forma reduzida (CysSH) a oxidada (dissulfeto, CysS-S), frequentemente envolvendo vários estados intermediários de oxidação, conforme ilustrado na Figura 1. Entretanto, há várias restrições cinéticas (p. ex., baixas constantes de velocidade de reação) e biológicas à oxidação direta e específica desses alvos por intermediários oxidantes (5). O conceito de *adaptador redox* surgiu a partir destas questões (5). Adaptadores são moléculas cineticamente sensíveis ao efeito de intermediários redox-ativos (p. ex., oxidação por peróxido de hidrogênio), capazes de transmitir este efeito a alvos específicos mediante interações proteicas. Como exemplo, peroxirredoxinas são rapidamente oxidadas por peróxido de hidrogênio e transmitem o dissulfeto a proteínas interatoras (6, 7). Um outro modelo de adaptador, menos estudado, são proteínas que modulam suas atividades por meio de alterações conformacionais oriundas de sua interação

com outras proteínas redox-modificadas, sendo capazes de exercer efeitos em alvos subcelulares específicos (8). Em ambos os casos, os principais exemplos de adaptadores redox são *tiol-proteínas*.

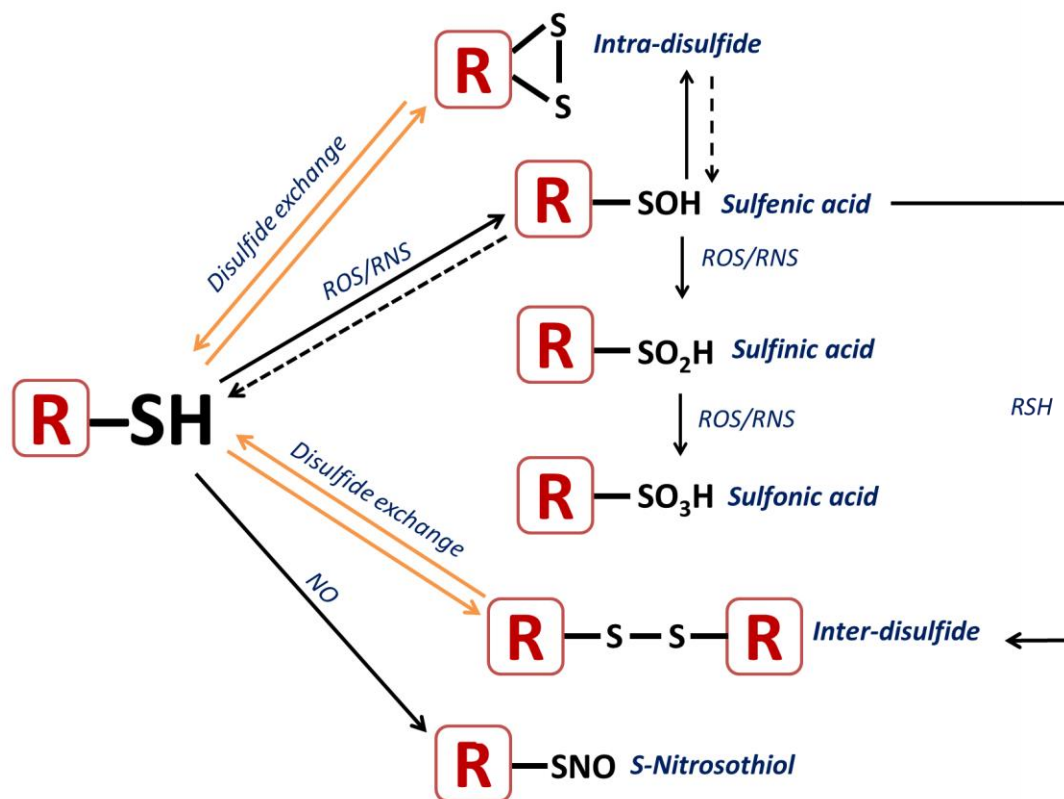


Figura 1. Painel de reatividade de tióis. Tióis podem gerar uma variedade de modificações que incluem as reversíveis como intra ou interdissulfetos, S-sulfenação, S-sulfinação, bem como modificações não reversíveis com S-sulfonação. O painel completo destas modificações é muito maior, porém destacamos apenas as principais. Figura retirada de Oliveira & Laurindo (4). ROS: espécies reativas de oxigênio, RNS: espécies reativas de nitrogênio; R-SH: tiol.

O entendimento do papel de tiol-proteínas e de seus estados intermediários de oxidação das cisteínas redox tem sido fundamental para melhor compreensão dos mecanismos de sinalização redox. Tiol-proteínas podem arbitrariamente ser classificadas como: 1) proteínas de sinalização tiol-reguladas (p. ex.: quinases, fosfatases, fatores de transcrição), que são alvos tiol-redox no contexto de sinalização celular; 2) proteínas armazenadoras de tiol (p. ex.: albumina plasmática) e 3) proteínas tiol-redox profissionais (p. ex.: glutatona peroxidases, peroxirredoxinas, glutatona transferases, tioredoxinas,

proteína dissulfeto isomerases), que compreendem as proteínas envolvidas como adaptadores em sinalização redox, capazes de modular alvos celulares específicos (4).

A superfamília da tiorredoxina é uma das mais importantes famílias de tiol-proteínas, envolvida em diversas funções homeostáticas básicas e em sinalização celular. Os membros desta família contêm usualmente um sítio redox-ativo com a sequência Cys-X-X-Cys, conhecido como motivo tiorredoxina, em que X representa um aminoácido indeterminado, capaz de influenciar potencial redox e cinética de reação da proteína contendo este motivo (9). Este domínio tem como característica importante um enovelamento específico (5 folhas β cercadas por 4 hélices α) (Figura 2B). O membro fundador desta super-família é a própria tiorredoxina e ainda inclui as proteínas formadoras de dissulfetos (DSBs e similares de procariotos), glutarredoxina, glutathione redutase, glutathione peroxidase e proteínas da família da Proteína Dissulfeto Isomerase (PDI).

1.1. Proteína Dissulfeto Isomerase: estrutura e função no retículo endoplasmático

As Dissulfeto Isomerases Proteicas (PDIs) são chaperonas com propriedades ditiol-dissulfeto oxido-redutase (10). A família das PDIs compreende 21 membros (11), sendo a PDI (55kDa, também chamada de PDIA1, codificada pelo gene *P4HB*) o membro fundador da família cujas atividades canônicas incluem: oxidação, redução ou isomerização de pontes dissulfeto durante o enovelamento proteico no lúmen do retículo endoplasmático (RE) (10). As PDIs variam em tamanho, arquitetura de domínios, expressão, localização e função, mas compartilham como característica estrutural comum a existência de domínios semelhantes à tiorredoxina (12), frequentemente (mas não obrigatoriamente) organizados em tandem com domínios redox-inativos também contendo o enovelamento tiorredoxina.

No caso da PDI, os 4 domínios tiorredoxina são ordenados sequencialmente como a, a', b, b', seguidos de um domínio c-terminal contendo a sequência KDEL (Lys-Asp-Glu-Leu) de recuperação para o RE (Fig. 2A). Os primeiros 17 aminoácidos correspondem à sequência de endereçamento para o RE (peptídeo de sinal) (13). A proteína, quando enovelada, assume um formato em “U”, no qual os domínios homólogos a e a' formam os

braços do “U” e contém os sítios catalíticos redox-ativos (motivo Cys-Gly-His-Cys) (Figura 2D). Os ditióis dos sítios a e a' constituem as cisteínas que interagem com os tióis ou dissulfetos nos substratos. Os domínios intermediários b e b' na base do “U” são cataliticamente inativos e ricos em resíduos hidrofóbicos, particularmente o bolsão hidrofóbico no domínio b', envolvido na ligação da PDI a substratos (9, 12, 14) (Fig. 2C). A região do *x-linker*, localizada entre os domínios a' e b', garante certo grau de relaxamento estrutural e mobilidade à proteína, gerando pelo menos duas conformações, “*capped*” e “*uncapped*”, que respectivamente restringem ou permitem a acessibilidade ao substrato (15, 16) (Fig. 2C). Além disso, o estado redox tem importante efeito na estrutura global da PDI: reduzida, assume estrutura mais compacta e oxidada, conformação mais aberta expondo domínios de ligação a substrato e incrementando sua atividade chaperona (Figura 2E) (14, 17).

PDI tem expressão ubíqua em diferentes tecidos e tipos celulares de mamíferos (10, 14). A concentração de PDI no RE é elevada, estimada em ca. 0.2-0.5 mM (18) e sua meia vida é alta, ca. 7 dias em fígado de rato (19). A principal função da PDI no RE é catalisar o enovelamento de proteínas nascentes por meio da introdução e isomerização de pontes dissulfeto. Estas proteínas-clientes são tipicamente destinadas à secreção e inserção em membrana plasmática, correspondendo a 1/3 de todas as proteínas celulares (10).

A introdução de pontes dissulfeto em proteínas nascentes pela PDI gera como subproduto a PDI reduzida, que necessita ser reoxidada para completar o ciclo catalítico. Esse processo de regeneração ocorre classicamente pela oxidoreductina do ER (Ero1), que é essencial à viabilidade celular em organismos como leveduras. A reoxidação da Ero1, por sua vez, é feita pela transferência de elétrons via FAD para o oxigênio molecular (14), gerando como subproduto peróxido de hidrogênio. Outra importante característica da PDI, além da sua atividade oxidoreductase ditiol/dissulfeto, é a sua atividade chaperona, que a permite distinguir entre substratos proteicos desenovelados, parcialmente enovelados e corretamente enovelados, mostrando alta afinidade para os primeiros (20). Esta propriedade, aliada à sua flexibilidade conformacional, faz da PDI uma chaperona de alta eficiência (21). Diferentemente das proteínas de choque térmico (HSPs), que vinculam sua

atividade chaperona ao consumo de ATP, as PDIs vinculam sua atividade chaperona à atividade tiol-oxidoreductase.

A PDI é superexpressa durante estresse do RE sustentado, embora não seja *per se* diretamente responsiva à *unfolded protein response* (13, 22). Uma outra rota pela qual a PDI contribui para proteostase é a degradação de proteínas mal-enoveladas pela via associada ao RE (ERAD), ao facilitar a translocação dessas proteínas do RE para o citoplasma, onde são subsequentemente degradadas via sistema ubiquitina-proteasoma (23, 24). PDI também está envolvida no controle de qualidade de proteínas destinadas à secreção ao mediar sua retenção no lúmen do RE para adequado processamento, um efeito que denominamos "*holdase-like*" (25). Este efeito é notório em proteínas com grande número de pontes dissulfeto, como a fibrilina-1 da matriz extracelular. O silenciamento da PDI em fibroblastos embrionários de camundongos com mutação de fibrilina-1 e Síndrome de Marfan acelera a secreção da fibrilina-1 mutante, escapando do controle de qualidade do RE e prejudicando a organização da matriz (25).

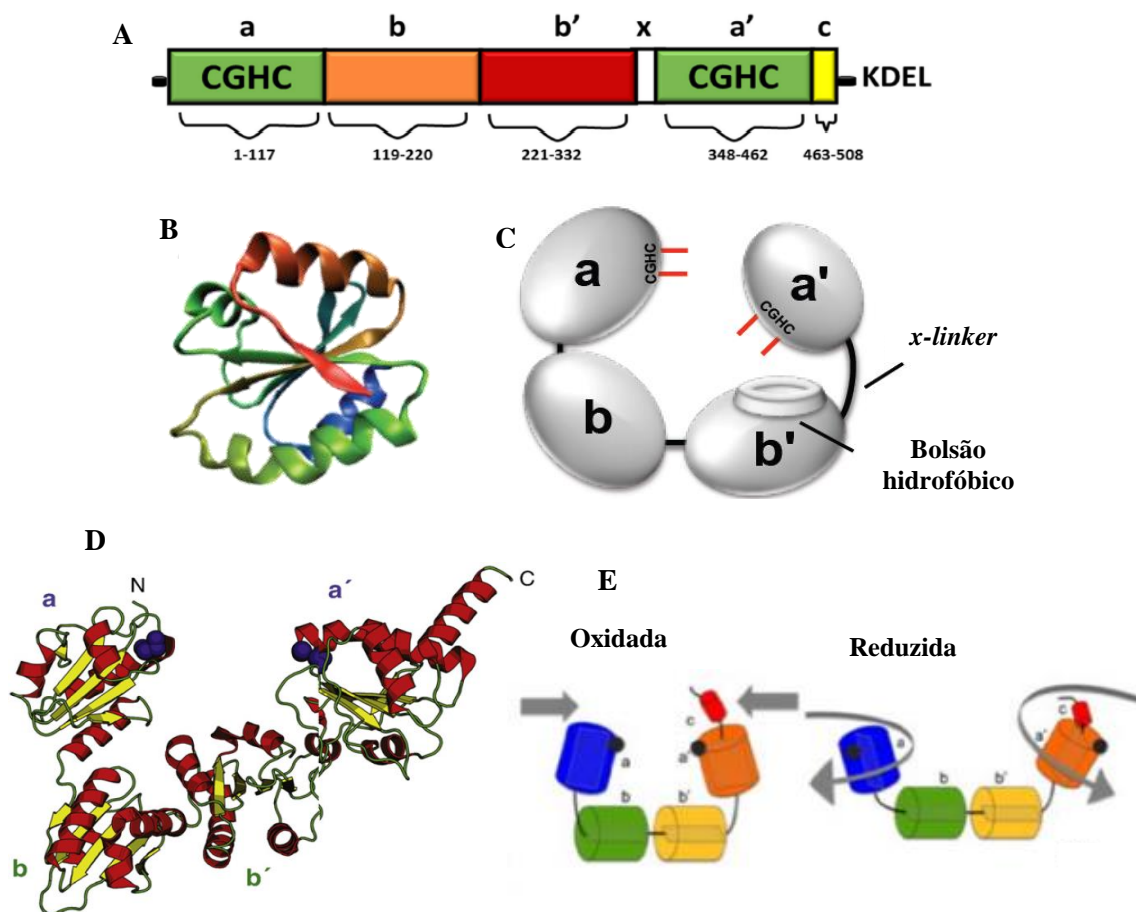


Figura 2. Proteína Dissulfeto Isomerase. **A.** Representação da estrutura linear da PDI. Os domínios semelhantes à tioredoxina são mostrados em verde, representando os domínios catalíticos a e a'. Os domínios cataliticamente inativos, b e b', estão mostrados em laranja e vermelho, respectivamente. O peptídeo sinal está mostrado em preto antes do domínio a. O *x-linker* (mostrado em branco), é responsável pelo formato de U da estrutura da PDI. O resíduo C-terminal está ilustrado em amarelo, seguido pela sequência KDEL de retenção no retículo. Figura retirada de Parakh & Atkin (13). **B.** Enovelamento típico dos domínios semelhantes à tioredoxina ($\beta\alpha\beta\alpha\beta\alpha$). Figura retirada de Flaumenhaft & Furie (26). **C.** Arquitetura da PDI. Os quatro domínios enovelados assumem o formato de "U". Os domínios a e a' têm as cisteínas catalíticas (mostradas em vermelho), responsáveis pela atividade oxidorredutase da PDI. Os substratos se ligam nos domínios b e b', e o bolsão hidrofóbico, primariamente responsável por esta ligação, está localizado no b'. O *x-linker*, um peptídeo flexível, conecta os domínios a' e b'. Figura retirada de Flaumenhaft & Furie (26). **D.** Estrutura tridimensional da PDI. As alfa-hélices são mostradas em vermelho, as folhas beta em amarelo e os *loops* em verde. As cisteínas nos sítios ativos estão representadas em esferas roxas. Figura retirada de Appenzeller-Herzog & Ellgaard (9). **E.** O estado redox da PDI altera sua configuração espacial. Quando reduzida, PDI assume configuração mais compacta e quando oxidada uma configuração mais aberta, expondo os domínios de ligação ao substrato. Figura retirada de Romer et al (27).

1.2. PDI fora do retículo endoplasmático: PDI epi/pericelular (*pecPDI*)

A presença da PDI em outros compartimentos celulares tem sido documentada de modo variável, incluindo a superfície celular/meio extracelular (*pecPDI*), mitocôndria, núcleo, citosol e meio extracelular, nos quais suas atividades parecem diferir das desempenhadas no lúmen do RE (8). É importante ressaltar que o grau de expressão da PDI fora do RE parece ser comparativamente mais baixo do que no lúmen do RE, embora não exista documentação clara para nenhum outro compartimento que não o meio extracelular, no qual a concentração de PDI é <2% do total em células endoteliais (28). Em paralelo, a variedade de implicações funcionais atribuída à *pecPDI* no contexto de trombose vascular (26), ativação de plaquetas (29), adesão celular (30), infecção viral (31-33) e remodelamento vascular anti-constritivo (34), indica um papel funcional relevante em diferentes tipos celulares.

Inúmeras evidências comprovam que PDI e outras chaperonas do RE podem ser secretadas para o meio extracelular na ausência de lise ou lesão celular (35, 36). A PDI é secretada por plaquetas, células endoteliais (CE) da aorta bovina, hepatócito de ratos, células B humanas, leveduras e linhagens de células leucêmicas, de fibrosarcoma e endotelial (37). No entanto, o mecanismo pelo qual a PDI alcança a superfície celular e/ou é secretada permanece desconhecido. Nosso grupo mostrou recentemente que a externalização da PDI é cerca de 75% Golgi-independente em CE (28) e totalmente independente do Golgi em células musculares lisas vasculares (38). Em CE a PDI é detectada em vesículas distintas das que contém fator von Willebrand (39), além disso, deficiência na biogênese de lisossomos e grânulos densos inibe a secreção de PDI em CE e plaquetas, associada a distúrbios de sangramento (40). Em plaquetas a PDI está localizada no sistema tubular denso (remanescente do RE dos megacariócitos) e é externalizada após ativação via processos que envolvem polimerização de actina, embora independente do receptor de ligação a proteína de fusão na membrana plasmática (Munc 13-4) (41).

Em CE, PDI da superfície celular forma complexos com a integrina $\alpha\beta3$, sugerindo um mecanismo pelo qual estas moléculas ficam retidas na membrana (42). O *pool* da PDI de superfície em CE também pode ativar $\beta1$ e $\beta3$ integrinas (33, 42) e regula a exposição de fosfatidilserina nestas células (43). CE ativadas convertem $\alpha\beta3$ para uma conformação

ativa de modo dependente da atividade da PDI potencialmente via troca tiol-dissulfeto (42). A maioria dos efeitos da *pecPDI* reportados até o momento estão associados à atividade redutase ou isomerase; nosso grupo, por outro lado, mostrou um efeito oxidase da *pecPDI* em tióis de integrinas após estímulos mecânicos em CE (28) ou células musculares lisas (44).

É importante ressaltar que as propriedades redox inerentes à PDI provavelmente modulam o seu modo de interação com os substratos. A baixa reatividade da PDI com o peróxido de hidrogênio ($k \approx 17,3 \pm 1,3 \text{ M}^{-1} \text{ s}^{-1}$) (45), muito inferior à esperada para sensores redox canônicos como peroxirredoxinas, cuja constante de reação é $k \approx 10^5\text{--}10^8 \text{ M}^{-1} \text{ s}^{-1}$ (46), indica que é pouco provável que a PDI atue como um sensor redox. Além disso, o baixo potencial redox da cisteína N-terminal dos domínios catalíticos da PDI (–180 mV) pode sustentar sua capacidade oxidativa, indicando que a PDI tem a capacidade de permanecer oxidada em um intervalo mais amplo de potenciais redutores do que, por exemplo, tiorredoxina (–270mV) (14). Em particular, a PDI tem um efeito chaperona que parece apoiar a organização de complexos proteicos, como as NADPH oxidases (47). Estas propriedades trazem duas implicações. Primeiro, a PDI provavelmente promove efeitos adaptadores ou moduladores redox direcionados de seus substratos ao invés de modulações inespecíficas (8). Em segundo lugar, no ambiente extracelular, em que a concentração de PDI é menor que a de seus substratos - ao contrário do que ocorre no lúmen do RE - a quantidade de PDI livre pode potencialmente ser afetada pelo tipo e capacidade de ligação dos substratos.

PDI tem papel essencial no acúmulo de plaquetas, formação do trombo e geração de fibrina após lesão vascular, efeito este reportado por diversos estudos (48). Especificamente, o sítio catalítico no domínio a' sustenta trombose arteriolar e tem papel chave na função de plaquetas e geração de fibrina (49). O efeito essencial da PDI presente na superfície de plaquetas para agregação está relacionado à modulação redox de diversos alvos, incluindo: a) ativação da integrina $\alpha\text{IIb}\beta\text{3}$ (50-52), b) ligação do colágeno à integrina $\alpha\text{2}\beta\text{1}$ (53) e c) modulação da atividade de GP1b α (54), trombospondina-1 (55, 56) e vitronectina (57). Recentemente, foi demonstrado que a PDI facilita a clivagem de dissulfetos alostéricos que regulam a função da integrina GP1b α , promovendo a interação

entre neutrófilos e plaquetas e levando à oclusão vascular sob condições trombo-inflamatórias (58). Além disso, diversos substratos da atividade tiol-redutase da PDI vêm sendo identificados pela técnica de captura cinética (*kinetic trapping*) e posteriormente caracterizados em eventos ligados à formação do trombo vascular (59-61).

Os papéis de PDIs na trombose vascular têm se destacado em estudos translacionais na busca por novas moléculas antitrombóticas, em que a lista de inibidores da PDI vem crescendo significativamente (16, 62, 63). A quercetina-3-rutinosídeo (rutina), conhecida por se ligar ao domínio b'-x da PDI (64) inibe a formação do trombo *in vivo* (65). Essa atividade antitrombótica dos inibidores da PDI, e o fato de a PDI da plaqueta afetar pouco a hemostasia fisiológica (66) sugerem a possibilidade da inibição da *pecPDI* como um novo e importante alvo antitrombótico. Recentemente, após uma busca de aproximadamente 348.000 compostos, foi identificada uma nova classe de inibidores reversíveis da PDI, chamada *bepristats* (*bep1a* e *bep2a*) que também se ligam no domínio b', especificamente no bolsão hidrofóbico. Estes inibem a atividade redutase da PDI no ensaio de agregabilidade da insulina com concentração inibitória mínima de $\sim 0.7\mu\text{M}$, mostrando maior eficiência de inibição comparado à rutina. Além disso, os *bepristats* também inibem a agregação plaquetária e a formação do trombo *in vivo* (16). A modulação terapêutica farmacológica da *pecPDI* e sua potencial aplicação em trombose associada à doenças foi recentemente descrita em um estudo utilizando a isoquercetina, um flavonóide de quercetina, que é mais facilmente absorvido que a rutina, e apresenta as mesmas propriedades inibidoras da PDI. Neste estudo multicêntrico de fase II, foi avaliada a eficácia da isoquercetina em reduzir hipercoaguabilidade em pacientes com câncer. A administração de 1000 mg/dia de isoquercetina aumentou a capacidade inibitória do plasma na atividade da PDI e reduziu significativamente a concentração plasmática de dímero-D, a geração de trombina dependente de plaquetas e os valores de P-selectina solúvel (67). Assim, este estudo é o primeiro a validar a eficácia de inibidores da PDI como moléculas anti-trombóticas. Pelo menos um ensaio clínico de fase III já está planejado.

Apesar do papel crítico da *pecPDI* na formação de trombos, atividade de plaquetas, bem como na biologia redox de células vasculares discutidos até o momento, existe pouca informação sobre a concentração circulante da PDI e outras tiol isomerases vasculares. Um

estudo reportou um *pool* de PDI no plasma de indivíduos saudáveis (57) e outros mostraram a secreção de PDI por plaquetas e CE, bem como em associação com micropartículas nos mesmos tipos celulares (68-70). No entanto, estes dados não estão associados a fenótipos específicos em situações fisiológicas ou de doenças. Recentemente, foi sugerido que PDI e outras tiol isomerases estão ausentes na circulação, como um mecanismo de supressão da formação de trombos; porém, após lesão vascular, a liberação de PDI a partir do endotélio lesado e plaquetas ativadas localmente reverteriam essa supressão promovendo trombose vascular por meio da modificação de substratos via reações tiol-dissulfeto (59).

Desta forma, considerando os efeitos fisiológicos e bioquímicos da PDI discutidos até o momento, a determinação precisa das concentrações de PDI plasmática em condições fisiológicas pode propiciar melhor entendimento de um potencial papel da PDI circulante como desencadeante ou marcador de eventos ligados à trombose e função endotelial.

2. Objetivos

2.1. Objetivo geral

Investigar a ocorrência e significância fisiológica de um *pool* circulante de PDI em indivíduos saudáveis.

2.2. Objetivos específicos

- 1) Caracterizar um *pool* detectável de PDI circulante em uma população de indivíduos saudáveis e validar a técnica de detecção (*Enzyme-Linked Immunosorbent Assay* – ELISA).
- 2) Investigar nesta população possíveis fatores associados aos valores de PDI plasmática.
- 3) Avaliar se as concentrações de PDI circulante podem ser marcadores/indicadores de perfis proteômicos plasmáticos e sua associação com fenótipo e respostas funcionais endoteliais potencialmente associadas com doença.

3. Métodos

3.1. Reagentes

Todos os reagentes foram adquiridos da Sigma-Aldrich, com exceção dos especificados a seguir. Kits de ELISA (*Human P4HB ELISA Pair Set*; SEK10827, lotes n°: KW05NO2203 e KW09NO1102) foram adquiridos da *SinoBiological*. Proteínas recombinantes ERp57 (TP305940) e ERp5 (TP301710) da *OriGene*. *TALON Affinity Collum Cobalt* para purificação da PDI recombinante foi adquirida da *Clonetch*. Meio *terrific broth*, Trizol da *Invitrogen*. colunas de dessalinização da *Amicon*. Membranas de nitrocelulose, *beads* magnéticas, coluna de sefarose PD-G25 da *GE Healthcare*. Anticorpos primários e diluições utilizadas para *western blot*: PDI, anticorpo de captura ELISA (1:500, *SinoBiological*), PDI clone RL90 (1:1000, *Thermo-Fisher*), ERp72 (1:1000, ab82587, *Abcam*), ERp57 (1:1000, ADI-SPA-585, *Enzo*). Anticorpos secundários obtidos da *Odyssey*. Anti-PDI para imunoprecipitação (SPA890, *Enzo*). Tubos para coleta de sangue foram obtidos da *Vacurette*. Eosina-isotiocianato, *EZ-Link Sulfo-NHS-(N-hydroxysuccinimido)-Biotin* da *Thermo-Fisher*. MPB [3-(*N*-maleimido-propionyl) biocytin] da *Molecular Probes*. Meios de cultura: *Dubelcos Modified Eagle Medium* (DMEM) e RPMI, da Soro fetal bovino *Gibco/Thermo-Fisher*. Coluna MARS Hu14 da *Agilent*. Placas e garrafas de cultura celular da *Corning*. RT² *First Strand kit* para extração de mRNA e kit RT² *Profile PCR array* (PAHS-038) foram obtidos da *Qiagen*.

3.2. População de estudo

Integraram o estudo 35 indivíduos voluntários saudáveis com características resumidas na Tabela 1. Os participantes não apresentavam histórico prévio de desordens agudas ou crônicas que incluíssem doenças: cardiovascular, digestiva, metabólica ou tumoral. Nenhum dos indivíduos fazia uso de tabaco ou medicações crônicas. O estudo foi aprovado pelo comitê de ética da Faculdade de Medicina da Universidade de São Paulo (número CAPPesq 3334/09/085) e os voluntários formalizaram a sua adesão ao estudo mediante a leitura e assinatura do termo de consentimento livre e esclarecido (Anexo A).

Um grupo adicional de indivíduos ($n=95$) com doença cardiovascular clinicamente evidente provenientes do programa Hipercol Brasil também foi avaliado. As amostras de

plasma utilizadas nas análises do presente estudo foram obtidas a partir de um biobanco previamente validado para estudos de análise genética de pacientes com Hipercolesterolemia Familiar (71, 72). A aprovação foi contemplada pelo mesmo comitê de ética e mesmo número CAPPesq.

3.3. ELISA (Enzyme-Linked Immunosorbent Assay)

As concentrações solúveis de PDI foram quantificadas usando o kit comercial *Human P4HB ELISA Pair set* de acordo com as instruções do fabricante, incluindo pequenas alterações. Resumidamente, placas de 96 poços foram cobertas com 100 µL/poço do anticorpo de captura diluído (2 µg/mL) e incubadas *overnight* a 4°C, seguidas pela adição das amostras (100 µL/poço) incubadas sob agitação por 2h a temperatura ambiente (T.A). Posteriormente, 100 µL/poço do anticorpo de detecção conjugado com peroxidase (HRP) (1µg/mL) foram incubados sob agitação por 2h a T.A. As placas foram lavadas com TBS-T (Tris 10 mM, NaCl 150 mM, tween-20 0,5%) após cada incubação. Na etapa final, 200µL/poço de uma solução de tetrametilbenzidina (TMB) foram adicionados e incubados por 30 min sob agitação. A densidade ótica de cada poço foi imediatamente determinada em leitor de placa em 450 nm. A concentração de PDI foi determinada de acordo com uma curva padrão (ajuste log/log). As amostras analisadas incluíram plasma pobre em plaquetas (não diluído), PDI recombinante (reduzida, oxidada, S-nitrosilada), lisado de células humanas (HUVEC, HCT116, HKE3), meio condicionado, micropartículas plasmáticas e as proteínas recombinantes ERp57 e ERp5.

3.4. Expressão e purificação da PDI recombinante

A sequência completa da PDI humana contendo uma sequência marcadora (*tag*) de histidina (PDI-His *tag*) foi clonada em plasmídeo pET28A e expressa em culturas de *Escherichia coli* (BL21 – DE3 –*codon plus*). As culturas cresceram em meio TB (*Terrific Broth*) contendo canamicina (50 µg/ml) a 37°C, sob agitação (250 rpm), até atingirem a densidade ótica (DO) 600 nm= 0,6. A expressão proteica foi induzida pela adição de 0,1 mM de IPTG, permanecendo por mais 4 horas sob agitação. As bactérias foram obtidas por centrifugação do meio (10.000×g, T.A, 10 min.). O sobrenadante foi descartado e o pellet

ressuspendido em tampão *Talon* (300 mM NaCl e 50 mM Na₂HPO₄, pH 7.0) gelado. As bactérias sofreram lise por sonicação em mistura contendo 2 mg/ml de lisozima. O lisado foi deixado sob agitação por 2h, 4°C. Em seguida o material foi centrifugado (10.000×g, 4°C, 20 min) para remoção do material insolúvel. As proteínas recombinantes contidas no sobrenadante foram purificadas seguindo o protocolo para purificação de proteína nativa do manual *TALON Affinity Collum Cobalt*. A eluição foi realizada em gradiente de imidazol e a proteína purificada submetida à dialise em tampão *Talon* para remoção do imidazol. As amostras obtidas foram analisadas em SDS-PAGE (densidade 12%). As bandas de proteína foram visualizadas através de coloração com Coomassie Blue (0,25% de *Coomassie brilliant blue*, 90% de etanol absoluto e 10% de ácido acético). A concentração inicial de proteínas foi determinada pelo método de Bradford e alíquotas de concentração conhecida foram armazenadas em freezer -80°C até o momento do uso.

3.5. Preparo da PDI reduzida, oxidada e S-nitrosilada

A PDI foi reduzida com 100 mM de glutathione (GSH) ou oxidada com 10 mM de diamida *overnight* a 4°C. Para o preparo da proteína nitrosilada, a PDI foi incubada com excesso molar de 200 vezes de S-nitrosoglutathione por 30 min, 37°C. Todas as amostras foram passadas em colunas de dessalinização (*Microcon YM-50*) para remoção do excesso dos produtos utilizados. As concentrações de PDI em solução foram quantificadas a 280 nm ($\epsilon=45.565 \text{ M}^{-1} \text{ cm}^{-1}$).

3.6. Expressão proteica por western blot

Quantidades iguais de proteínas obtidas a partir de lisados celulares (HUVEC, HCT116, HEK3) ou meio condicionados (MC) foram submetidas a SDS-PAGE em gel de poliacrilamida (densidade 12%) e transferidas para membrana de nitrocelulose em sistema de transferência úmida. As membranas foram incubadas por 1h em tampão de bloqueio (TBS-T, 0,1% tween-20 com 5% de leite desnatado). A identificação das proteínas de interesse foi realizada pela incubação com os seguintes anticorpos primários: anti-PDI (anticorpo de captura do kit de ELISA *Human P4HB Pair Set* ou clone RL90), anti-ERp72, anti-ERp57, anti- β actina *overnight*, 4°C. Após sucessivas lavagens com TBS-T, as

membranas foram incubadas com os anticorpos secundários fluorescentes adequados por 1h, e o sinal foi detectado usando o sistema LI-COR (*Odyssey*).

3.7. Obtenção de plasma pobre em plaquetas

Sangue venoso foi coletado em tubos de EDTA e o plasma pobre em plaquetas foi separado por centrifugação a $2.500\times g$ durante 15 min, 4°C . Todas as amostras foram armazenadas a -80°C até o momento do uso.

3.8. Imunoprecipitação

Pools de plasmas recém coletados isolados a partir de três doadores (10 mL) foram diluídos 1:1 (v/v) em tampão de lise (50 mM Tris-HCl; 150 mM NaCl; 1 mM EDTA, pH 7,4; 1% Triton-X 100) suplementado com inibidores de protease (1 mM PMSF, 1 $\mu\text{g}/\text{mL}$ leupeptina e aprotinina), fosfatase (50 mM fluoreto de sódio, 2 mM ortovanadato de sódio, 10 mM pirofosfato de sódio) e 10 mM MgCl_2 . As amostras foram incubadas com anticorpo anti-PDI produzido em coelho (10 μg) *overnight*, 4°C , sob agitação, seguidas de incubação com 50 μL de *beads* magnéticas cobertas com proteína G por 4h, 4°C . Posteriormente as *beads* foram lavadas com o mesmo tampão de lise para remoção de material contaminante e incubadas com tampão de amostra (50 mM Tris-HCl, pH: 6,8; 5% 2-mercaptoetanol; 2% SDS; 0,05% bromofenol; 10% glicerol) por 15 min no gelo e fervidas por 5 min (o ciclo foi repetido por 3 vezes). As amostras foram submetidas a SDS-PAGE em gel de poliacrilamida (densidade 12%). As proteínas foram transferidas para membrana de nitrocelulose, seguidas do bloqueio da membrana por 2h (TBS-T, 10% de leite desnatado). As membranas foram incubadas *overnight*, 4°C com anticorpo primário anti-PDI, clone RL90, produzido em camundongo e em seguida lavadas por três vezes com TBS-T e incubadas com anticorpo secundário adequado. As membranas foram escaneadas utilizando o sistema LI-COR (*Odyssey*).

3.9. Ensaio de atividade tiol-redutase da PDI no plasma

O ensaio descrito a seguir utiliza a sonda di-eosina-glutationa dissulfeto (GSSG) para medir a atividade de moléculas com potencial redutor. A reação baseia-se na redução

do dissulfeto de glutationa (GSSG) (Figura 3A) – este composto, quando ligado covalentemente a duas moléculas de eosina em dois grupos amino livres (di-eosina-GSSG) (Figura 3B) funciona como uma sonda eficiente para medir a atividade tiol redutase, adicionando alta sensibilidade ao ensaio, capaz de detectar concentrações nanomolares da PDI, por exemplo (73, 74). A di-eosina-GSSG (Figura 4B) é uma molécula não fluorescente devido à proximidade das duas moléculas de eosina (“*self-quenching*”), no entanto, após redução (por redutases ou por concentrações milimolares de agentes redutores), a sonda apresenta um aumento na fluorescência de aproximadamente 70 vezes. Este ensaio tem sido amplamente empregado para medir a atividade tiol redutase da PDI recombinante (16, 73), bem como, a atividade da tioredoxina redutase em amostras de soro, plasma e lisados de linfócitos (74).

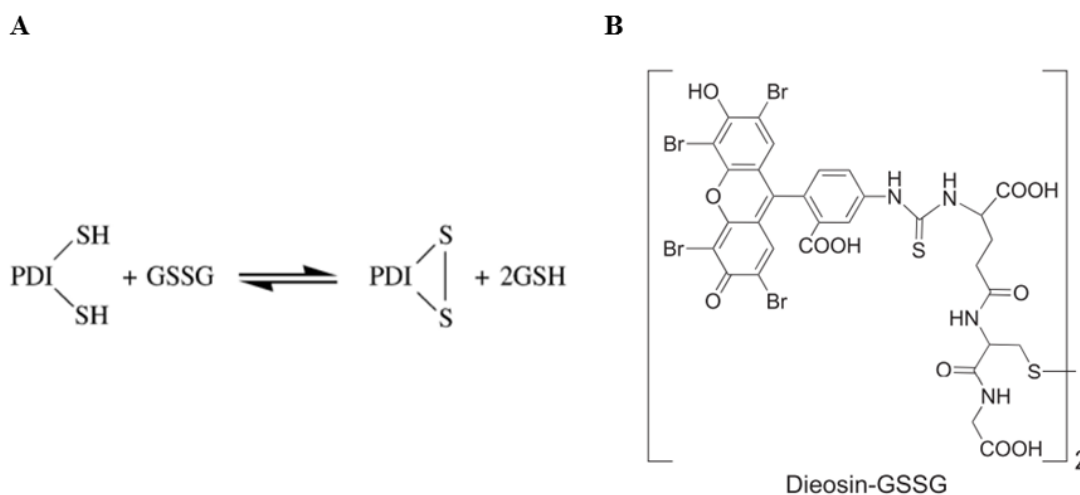


Figura 3 – Representação da reação tiol redutase da PDI. **A.** Redução da glutationa oxidada (GSSG) pela PDI reduzida. Figura retirada de Raturi & Mutus (73). **B.** Sonda fluorescente, Di-E-GSSG. Figura retirada de Foster & Thorpe (75).

A sonda foi preparada incubando 200 μ M GSSG com 2 mM eosina-isotiocianato em tampão fosfato 0,1 mM (PE) contendo 2 mM EDTA (pH 8,5) a T.A, *overnight*, sob proteção da luz (16, 73). A mistura foi passada através de uma coluna PD-G25 para remoção de produtos não ligados e diferentes frações foram coletadas. O aumento na intensidade fluorescência da sonda (Excitação: 520 nm, emissão: 545 nm) foi calculada usando amostras incubadas por 30 min com tampão PE (controle) ou 20 mM de DTT (potencialmente reduzidas) utilizando fluorímetro de placa (SpectraMax-M5). As frações

que apresentaram aumento de fluorescência >5 vezes após redução com DTT foram armazenadas a -20°C para uso posterior. A concentração da di-eosina-GSSG foi determinada usando o coeficiente de absorção molar para eosina ($\epsilon=56.000\text{ M}^{-1}\text{ cm}^{-1}$) a 525 nm em tampão PE (74). A atividade redutase no plasma foi obtida pela incubação de 0,5 mL de plasma recém coletado em tampão de ensaio (PE pH: 7,4 contendo 2 mM de EDTA), 5 μM DTT e 150 nM de di-eosina-GSSG na presença ou ausência de 60 μM de rutina. O aumento de fluorescência foi determinado por 20 min como descrito acima. A redução de 150 nM de di-eosina-GSSG na presença de 5 μM de DTT foi utilizada como controle negativo (branco). Cinco experimentos independentes foram realizados e cada amostra foi analisada em duplicata. Os resultados foram normalizados pela fluorescência total do plasma (controle) em unidades arbitrárias de fluorescência (RFU) e representada como porcentagem de atividade redutase.

3.10. Separação e lise de micropartículas plasmáticas

O protocolo foi realizado como descrito previamente (76). Brevemente, os plasmas (5 mL) foram diluídos 1:1 (v/v) em PBS (NaCl 140 mM; KCl 2,7 mM; Na_2HPO_4 anidro 1,5 mM; pH: 7,4) e centrifugados por 30 min a $2000\times g$, 4°C . Os sobrenadantes foram transferidos para tubos de ultracentrifuga sem o pellet de contaminantes e ultracentrifugados a $12.000\times g$ por 45 min, 4°C . Os sobrenadantes foram transferidos para tubos novos e ultracentrifugados por 2h a $110.000\times g$, 4°C . Os sobrenadantes foram descartados, os pellets invisíveis foram ressuspensos em 1 mL de PBS e posteriormente diluídos para o volume final do tubo utilizado (10 mL). As suspensões resultantes foram filtradas (filtro 0.22 μm) e novamente ultracentrifugadas a $110.000\times g$ por 70 min, 4°C (esta etapa de centrifugação foi repetida mais uma vez). Os pellets finais foram ressuspensos em 100 μL de PBS e armazenados a -80°C . As micropartículas isoladas foram acrescidas com tampão de lise (Hepes 20 mM; NaCl 150mM; glicerol 10%; triton 1%; EGTA 1 mM; MgCl_2 1,5 mM) na presença de inibidores de protease (1 mM PMSF, 1 $\mu\text{g/ml}$ aprotinina e leupetina) e incubadas por 30 min a 4°C , posteriormente sonicadas e em seguida centrifugadas 10 min a 14.000 rpm, 4°C para retirada dos *debris*. Todas as amostras foram armazenadas a -80°C até o momento da dosagem.

3.11. Ensaio para detecção do estado redox da PDI no plasma utilizando sondas biotiniladas

Para investigar o estado redox da PDI no plasma, alíquotas de plasma recém coletado (200 μ L) foram separadamente biotiniladas com excesso de MPB [3-(N-maleimido-propionyl) biocytin] (4 mM), que se liga a tióis livres ou EZ-Link Sulfo-NHS-(N-hydroxysuccinimido)-Biotin (4 mM), que se liga a grupos amino de proteínas, protegidos da luz, sob agitação, T.A. por 30 min. Todos os reagentes foram preparados em tampão HBS (20 mM HEPES; 0,14 M NaCl; 1,5 mM CaCl_2 ; 4 mM KCl; 0,5 mM Na_2HPO_4 , pH: 7,4) como descrito previamente (77). Em seguida, as amostras foram diluídas em 1 mL de HBS e incubadas da mesma maneira por 10 min adicionais. MPB e Sulfo-NHS não ligados foram removidos por precipitação utilizando acetona. Os *pellets* proteicos foram ressuspensos em 250 μ L de PBS-Tween/20 (0,05%) e constituíram as amostras de plasma biotiniladas. Como controle não biotinilado, amostras de plasma foram incubadas em tampão HBS na ausência das sondas ligadas à biotina. Como controle interno para analisar variações na biotinação de proteínas plasmáticas com MPB ou Sulfo-NHS entre os ensaios, um *pool* de plasmas (amostra controle padrão) foi utilizado em todos os ensaios. Todas as amostras e controles foram preparadas concomitantemente. Para quantificação da PDI nestas amostras, o ensaio de ELISA descrito anteriormente foi adaptado. Primeiramente, placas de 96 poços foram cobertas com o anticorpo de captura do kit de ELISA (*Human P4HB Pair Set*) e incubadas *overnight*, 4°C. Antes da adição das amostras biotiniladas e controles, as placas foram lavadas 3 vezes com TBS-T e bloqueadas por 1h com TBS-T/2% BSA (albumina). As amostras foram então adicionadas na placa (100 μ L/poço) em duplicata e incubadas por 2h sob agitação, T.A. Após este período as placas foram lavadas 3 vezes com TBS-T e o anticorpo monoclonal anti-biotina conjugado com peroxidase (1:200) (Clone BN-34) foi adicionado por 2h, sob agitação, T.A. Finalmente os poços foram lavados 3 vezes com TBS-T e uma solução de TMB (200 μ L/poço) foi adicionada por 30 min sob agitação, T.A. A densidade óptica de cada poço foi imediatamente determinada em leitor de placa a 450 nm (SpectraMax-340, Molecular Devices). A marcação de aminas totais (Sulfo-NHS bitoina) foi considerada como PDI total e a marcação de tióis livres com MPB foi considerada como a fração reduzida da PDI.

3.12. Cultura de células

Células endoteliais de cordão umbilical humano (HUVEC) imortalizadas por sucessivas passagens foram cultivadas em meio RPMI suplementadas com 10% de soro fetal bovino (FBS) contendo antibióticos (estreptomicina 100 mg/L e penicilina 25 mg/L) e 10 mM HEPES em estufa a 37°C em atmosfera de 95% O₂/5% CO₂. As linhagens celulares de carcinoma de cólon HCT116 e HKE3 (doadas pelo Dr. Walter Kolch, Universidade de Dublin) foram mantidas em meio DMEM suplementado com 10% de FBS sem antibióticos em estufa a 37°C em atmosfera de 95% O₂/5% CO₂.

3.13. Incubação de células endoteliais com plasma

Células endoteliais HUVEC foram subcultivadas, lavadas com PBS, tratadas com tripsina e ressuspendidas em RPMI suplementado com 10% FBS para semeio em placas de 100mm na densidade de $2,5 \times 10^6$ células. Após cultivo por 22-24h, as células foram lavadas com PBS e cultivadas por 24h adicionais onde FBS (5%) foi substituído por 5% de um *pool* plasmático de indivíduos com alta ou baixa concentração circulante de PDI. Os plasmas ricos ou pobres em PDI foram obtidos a partir de três doadores em cada grupo (volumes iguais de plasmas isolados de cada doador compuseram os *pools*). A concentração utilizada (5%) foi determinada a partir de experimentos prévios de validação; a incubação de células endoteliais com RPMI suplementado com 10% dos *pools* plasmáticos se associou com significativa perda de adesão das células, embora as mesmas tenham permanecido viáveis após as 24h de cultivo.

3.14. Ensaio de viabilidade celular

Após cultivo por 22-24h as células HUVEC foram lavadas com PBS e cultivadas por 24h adicionais, nas quais o SFB (5%) foi substituído por 5% de um *pool* plasmático de indivíduos com alta ou baixa concentração circulante de PDI. Após este período, as células foram avaliadas quanto à viabilidade usando o teste de exclusão do azul de tripan. Células descoladas foram misturadas com uma solução de azul de tripan (0,4%) e analisadas em contador de células automático (*Countess II FL, Thermo-Scientific*). As células marcadas

em azul foram identificadas como não viáveis, uma vez que retiveram o corante internalizado, enquanto as células viáveis foram capazes de excluí-lo.

3.15. Análise proteômica

3.15.1. Preparo de amostra e análise UPLC-HDMSE

3.15.1.1. Amostras de plasma

Quarenta microlitros de plasma obtidos a partir de 6 doadores (3 plasmas pobres em PDI; 3 plasmas ricos em PDI) foram diluídos em 120 µL de tampão (solução A) proveniente do protocolo experimental do sistema em coluna MARS Hu14, utilizado para depleção de proteínas altamente abundantes no plasma. A cromatografia líquida de imunoafinidade foi realizada de acordo com Garcia et al, (78).

3.15.1.2. Meio condicionado (MC) de HUVEC

Células HUVEC foram cultivadas e tratadas como descrito acima na sessão ‘Incubação de células endoteliais com plasma’. Para obtenção do MC, o meio de cultura foi removido e as células cuidadosamente lavadas com PBS para posterior incubação com 6 mL de RPMI sem FBS. Após 8h em cultura, os sobrenadantes foram coletados e os *debris* celulares removidos por centrifugação (1.400 rpm, 10 min, T.A) seguidos por concentração utilizando *SpeedVac*. O meio concentrado foi estocado a -80°C até o momento do uso. A concentração de proteínas foi determinada por ensaio de Bradford. As amostras (50 µg/amostra) foram adicionadas em tampão de amostra, fervidas por 5 min (95°C) e então submetidas a SDS-PAGE (densidade do gel 12%). As canaletas correspondentes a cada amostra foram destacadas no gel e divididas em >20 fragmentos. As proteínas contidas nos fragmentos foram tripsinizadas (*in gel*) a 37°C *overnight*. Para extração dos peptídeos, 50 mM (NH₄)CO₃ (50 µL), 2×50µL 50% acetonitrila, 5% ácido fórmico foram adicionados aos fragmentos de gel. A solução de extração foi reduzida a um volume <20µL usando *SpeedVac*.

3.15.2. Método de aquisição (espectrometria de massa)

Peptídeos (0,5 µg) foram automaticamente carregados em gerenciador de amostras de alta performance em um sistema UPLC classe-M (*Waters Corporation, Milford, MA*), acoplado *online* a um espectrômetro de massa *Synapt G2-Si* (*Waters Corporation, Milford, MA*). Os peptídeos capturados foram eluídos em gradiente de acetonitrila 7% a 40% (v/v) por 52 min em uma velocidade de fluxo de 0,5 µL/min e analisados diretamente no *Synapt G2-Si*. Os peptídeos foram ionizados usando uma fonte *nano-electrospray* em modo positivo. Os resultados foram obtidos em modo independente de dados (DIA) combinado com o modo de aquisição através de mobilidade iônica (HDMSe). Os parâmetros do espectrômetro de massas foram definidos de acordo com Cassoli et al, (79).

3.15.3. Identificação e quantificação de proteínas

As proteínas foram identificadas e quantificadas usando algoritmos do *software Progenesis QI Proteomics* (*Waters Corporation*). Os dados foram pesquisados em relação ao banco de dados proteômicos de humanos UniProt: para amostras de plasma, foi utilizado o banco de dados completo (versão: 2016_06) e para amostras de células HUVEC foi utilizado o banco de dados revisado (versão: 2017_10). Os parâmetros a seguir foram considerados para a identificação de peptídeos nas amostras de plasma: 1) Digestão por tripsina com no máximo uma clivagem perdida; 2) modificações variáveis por oxidação (M) e modificações fixas por carbamidometil (C); 3) taxa calculada de falsas descobertas (FDR) inferior a 4% em todas as condições e replicatas; 4) erro de massa inferior a 20 ppm. Um ou mais fragmentos iônicos por peptídeo, três ou mais fragmentos por proteína e um único ou diversos peptídeos por proteína foram necessários para a correspondência iônica. Os seguintes parâmetros foram considerados para identificar peptídeos em amostras de HUVEC: 1) Digestão por tripsina com no máximo uma clivagem perdida; 2) modificações variáveis por oxidação (M) e modificações fixas por carbamidometil (C); 3) taxa calculada de falsas descobertas (FDR) inferior a 1% em todas as condições e replicatas; 4) erro de massa inferior a 20 ppm. Dois ou mais fragmentos iônicos por peptídeo, cinco ou mais fragmentos por proteína e um único ou mais peptídeos por proteína foram necessários para

correspondência iônica. As identificações que não se encaixaram nesses critérios foram descartadas. A lista final de proteínas considerou apenas aquelas identificadas por pelo menos 2 peptídeos. Além disso, as diferenças entre os grupos foram analisadas usando ANOVA de única via e apenas valores $p < 0,05$ foram considerados significativos. Queratinas foram excluídas dos dados.

3.15.4. Análise de enriquecimento de vias

O software *Metacore* (Thomson Reuters, MI, USA) foi utilizado para análise funcional de enriquecimento de vias e geração de redes biológicas. Vias e processos biológicos foram considerados significativos quando o $p < 0,05$.

3.16. Agregação plaquetária

Plasma rico em plaquetas (PRP) foi obtido de voluntários saudáveis que não faziam uso de medicações anti-plaquetárias por pelo menos 10 dias previamente à coleta de sangue. As amostras de sangue foram coletadas utilizando citrato trisódico 3,2% como anticoagulante (sangue/anticoagulante 9:1 v/v) e submetidas a centrifugação $190 \times g$ por 15 min, T.A. O PRP resultante foi submetido à contagem de plaquetas em contador hematológico automático e o número de plaquetas ajustado para $2-3 \times 10^8 / \text{mL}^{-1}$. Para agregação plaquetária, alíquotas de 400 μL de PRP foram adicionadas em agregômetro pré-aquecido a 37°C e as plaquetas estimuladas com ADP (10 μM), colágeno (5 $\mu\text{g}/\text{mL}$) ou ionóforo de cálcio-A23187 (20,5 μM) por 5 min, 37°C , sob agitação constante em agregômetro *Chrono-log*. Os resultados em duplicata foram analisados utilizando o software *Aggro/Link* (versão 4.75), utilizando a calibração do plasma pobre em plaquetas como 100% de transmitância de agregação. Os resultados foram expressos como porcentagem de agregação plaquetária. Amostras de sangue total foram coletadas utilizando citrato trisódico 3,2% como anticoagulante (sangue/anticoagulante 9:1 v/v) e posteriormente alíquotas de 400 μL de sangue total foram acrescidas de 400 μL de solução salina e adicionadas em agregômetro pré-aquecido a 37°C . As amostras foram estimuladas com colágeno (25 μM) ou solução salina (controle negativo) por 10 min, 37°C , sob

agitação constante. Os resultados em duplicata foram analisados utilizando o mesmo software e expressos como porcentagem de agregação plaquetária.

3.17. Expressão de RNA mensageiro por PCR em tempo real

HUVEC foram cultivadas e tratadas como descrito acima na sessão ‘Incubação de células endoteliais com plasma’. Posteriormente, o meio de cultura foi removido, as células cuidadosamente lavadas com PBS e homogeneizadas em 1 mL de *Trizol* para extração do RNA mensageiro (mRNA). O mRNA foi quantificado medindo-se a absorbância em 260 nm (*Nanodrop ND2000, Thermo-Fisher*), a pureza pela relação da absorbância entre 260/280 nm e a integridade das amostras através da análise de bandas de RNA ribossomal (28S e 18S) em gel de agarose (densidade 0,8%) contendo brometo de etídeo. Os mRNA (1,5 µg) foram convertidos em cDNA utilizando o kit *RT² First Strand*. A biblioteca de cDNAs gerada foi utilizada para análise da expressão gênica de diversos genes por PCR (reação em cadeia da polimerase) em tempo real utilizando a técnica de *PCR array*. As reações foram realizadas em sistema *StepOnePlus*. (*Applied Biosystems*). Foi mensurada a expressão gênica de 84 genes (listados em Anexo D) usando o kit *RT² Profile PCR array* (PAHS-038) de acordo com as especificações do fabricante. O *PCR array* foi realizado a partir de 3 experimentos independentes. Quantidades relativas de mRNA foram calculadas pelo método $\Delta\Delta C_t$ utilizando a expressão da β -2-microglobulina como gene normalizador.

3.18. Ensaio de adesão celular

Placas de 96 poços foram cobertas com fibronectina (5 µg/mL) ou colágeno tipo-I (10 µg/mL) *overnight*, 4°C e bloqueadas por 2h com PBS/BSA 1%, 37°C. Células HUVEC cultivadas e preparadas como descrito acima na sessão ‘Incubação de células endoteliais com plasma’ foram lavadas com PBS e descoladas com PBS-EDTA (500 µM, pH 7,4). Em seguida, as células foram ressuspensas em RPMI contendo 0,2% BSA (tampão de adesão) e adicionadas aos poços (150 µL/poço) na densidade 4×10^4 células/poço. As células foram incubadas em estufa, 37°C, para permitir a adesão por 1h quando o substrato de matriz foi a fibronectina e por 4h30min quando o substrato foi o colágeno. Após os períodos determinados, as culturas foram lavadas gentilmente 3 vezes com PBS para a

retirada das células não aderidas e as remanescentes que aderiram foram fixadas com glutaraldeído 2% por 30 min, T.A. Após este período, as placas foram lavadas 3 vezes com PBS e coradas com cristal violeta 0,1% (30 min, T.A) seguidas por solubilização com SDS 1%. A densidade óptica de cada poço foi determinada em leitor de placa em 600 nm (*SpectraMax-340, Molecular Devices*).

3.19. Ensaio de migração celular (*Wound healing*)

A migração de células endoteliais foi investigada por ensaio de *wound healing* como descrito previamente (80). Resumidamente, células HUVEC foram semeadas em placas de 24 poços (2×10^5) e após 48h as monocamadas confluentes foram incubadas com HBSS (*Hanks' Balanced Salt solution*) sem Ca^{2+} e Mg^{2+} por 5 min. Uma lesão foi feita nas monocamadas celulares de cada poço com o auxílio de uma ponteira estéril (500 μm de diâmetro). As culturas foram lavadas 2 vezes com HBSS para remoção dos *debris* celulares. As células foram então incubadas com RPMI suplementado com 5% de FBS ou 5% de plasmas ricos ou pobres em PDI. A migração celular foi observada em microscópio (*Axiocvert 200, Zeiss*) e as imagens capturadas nos tempos 0h, 8h, 16h e 24h de incubação (3 campos/poço) com objetiva de 5x. A superfície coberta foi medida usando o *software Image-J*. A distância de migração celular foi determinada medindo a área coberta após indução da ferida e subtraindo esse valor da largura inicial da mesma. Os ensaios foram realizados em 4 replicatas.

3.20. Análise estatística

Os dados com distribuição normal e não-normal foram representados como média \pm erro padrão da média e mediana com intervalos interquartis, respectivamente. Diferenças entre os grupos foram analisadas usando teste t de *Student* para comparar dois grupos ou ANOVA de uma via com teste *post-hoc* de *Turkey* ou *Mann-Whitney*. Coeficientes de correlação foram gerados de acordo com o método de *Spearman*, $p < 0,05$ foi considerado significativo. Os gráficos e testes foram realizados utilizando o software *GraphPad Prism 7.0*. Todas as análises foram realizadas usando pelo menos 3 experimentos independentes com >2 replicatas quando especificado. Foram realizados testes de normalidade (*Shapiro-*

Wilk e *D'Agostino-Pearson Omnibus*) seguidos pela distribuição de frequência para os valores da PDI no plasma usando os parâmetros do Prisma. Análise de regressão linear foi utilizada para estimar a associação entre a concentração plasmática da PDI e os fatores de risco tradicionais em pacientes do banco de plasma.

4. Resultados

4.1. Validação do ensaio de ELISA para detecção da PDI

A concentração circulante da PDI foi investigada utilizando a técnica de ELISA (*Enzyme-Linked Immunosorbent Assay*), devido ao seu grau geralmente alto de especificidade e sensibilidade, bem como à existência de concentrações elevadas de proteínas constitutivas como albumina, globulinas e outras no plasma, fato que dificulta a utilização de outras técnicas. Após testar diferentes abordagens, que se mostraram insuficientemente sensíveis, uma busca criteriosa por kits de ELISA para PDI com alto grau de sensibilidade foi realizada e um kit de ELISA produzido pela empresa *SinoBiological* (*Human P4HB Pair Set*) foi selecionado. O kit, apesar de estar disponível comercialmente, ainda não havia sido validado na literatura. Por esta razão realizamos testes para investigar a especificidade e eficiência do ensaio quanto à detecção da PDI. A precisão inter e intra-ensaio foi obtida pela determinação do coeficiente de variação entre as amostras e os resultados indicaram um ensaio eficiente e capaz de detectar concentrações de PDI que variaram de 47 a 3000 pg/mL, os limites mínimo e máximo de detecção deste ensaio, respectivamente. (Figura 4A e B). Usando proteína recombinante foram geradas curvas de PDI confirmando sua precisa detecção (Figura 4C). O ensaio foi igualmente capaz de detectar PDI em diferentes estados redox (reduzida, oxidada e S-nitrosilada) (Figura 4D).

Adicionalmente, quantificamos a PDI intracelular em lisados de células humanas (HUVEC, HCT116 e HKE3) (Figura 5A), em paralelo à análise de *western blot* nas mesmas amostras usando o anticorpo de captura do kit de ELISA em questão. Como mostrado na Figura 5B, o anticorpo marcou banda única da PDI com peso molecular esperado (55 kDa), sem bandas inespecíficas que poderiam ser remanescentes de marcação para outras PDIs. Este resultado confirmou a especificidade do anticorpo de captura em detectar apenas a PDI. Também foi investigado se outros membros da família da PDI poderiam ser detectados pelo kit de ELISA por uma possível reatividade cruzada. Embora não seja possível testar todos os membros desta família, foram escolhidas as proteínas ERp57 (PDIA3) e ERp5 (PDIA6), uma vez que essas isoformas têm 33,6% (51,0%) e 23,5% (31,4%) de identidade (e similaridade entre parênteses) com a PDI, respectivamente (11) e a ERp57 tem arquitetura de domínios idêntica à da PDI. Além disso, ambas as

isoformas também são encontradas em células vasculares e possuem conhecida importância funcional na superfície de plaquetas e CE, compartilhando com a PDI papéis importantes na formação do trombo após lesão vascular (15). Os resultados apresentados na Figura 5C mostram que o ensaio reconhece apenas a PDI, uma vez que nenhum sinal foi detectado quando ERp57 e ERp5 foram testadas utilizando concentrações idênticas de proteína recombinante.

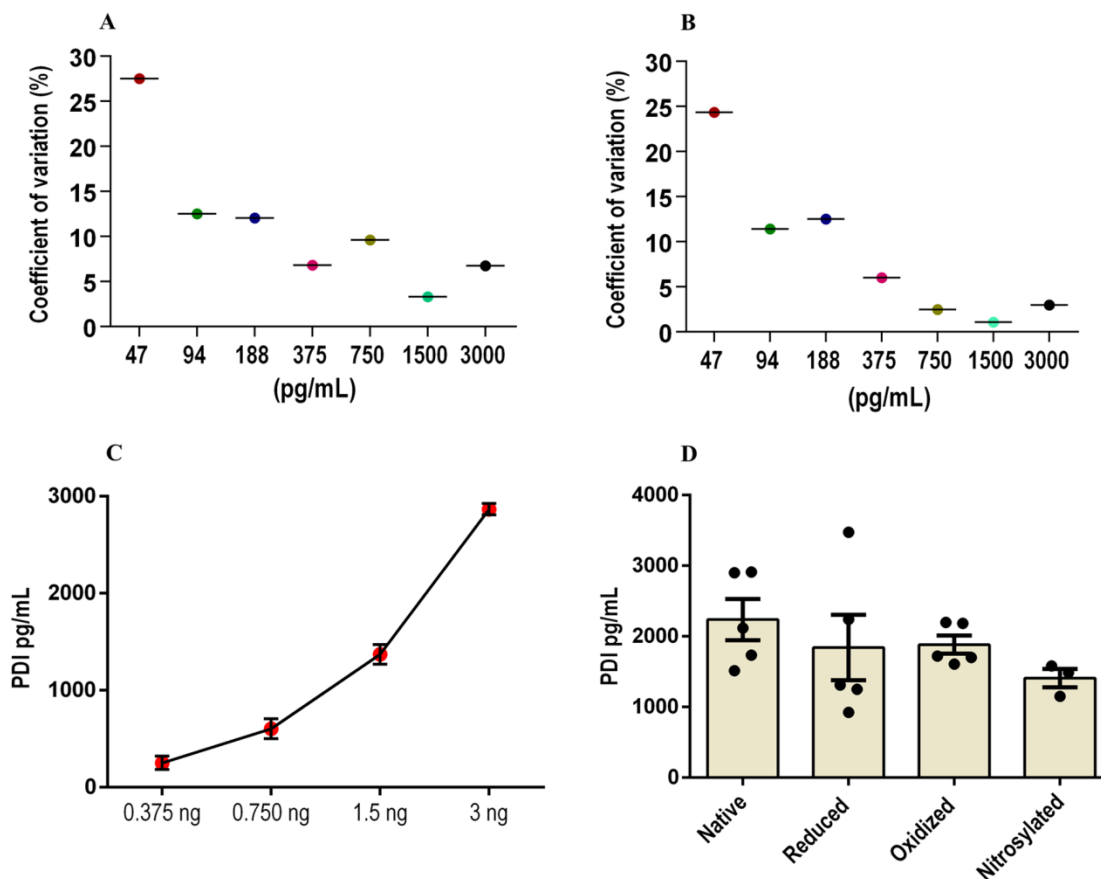


Figura 4 – Detecção da PDI recombinante pelo kit de ELISA ‘Human P4HB Par Set. A. Coeficiente de variação para precisão inter-ensaios. Curvas-padrão foram preparadas em duplicata a partir de padrões fornecidos pelo fabricante preparadas e analisadas em 4 ensaios independentes. **B.** Coeficiente de variação para precisão intra-ensaios. Curvas-padrão foram preparadas em duplicata a partir de padrões fornecidos pelo fabricante e analisadas simultaneamente na mesma placa. As concentrações de PDI variaram de 47–3000 pg/mL. O coeficiente de variação (em porcentagem) foi calculado a partir das médias dos 4 ensaios simultâneos, para cada um dos pontos da curva-padrão da PDI. As barras representam a média. **C.** Uma concentração conhecida de PDI recombinante (3 ng/mL) foi diluída serialmente até a concentração final de 0,375 ng/mL e sua detecção avaliada de acordo com os parâmetros estabelecidos pelo kit utilizado. As barras representam a média \pm erro padrão ($n=4$). **D.** PDI nativa, reduzida, oxidada e nitrosilada (2 ng/mL) foram dosadas em ensaio de ELISA. PDI recombinante foi reduzida por 10 mM de GSH, oxidada com 10 mM diamida e S-nitrosilada com S-nitrosoglutationa. As barras representam média \pm erro padrão ($n=3-4$).

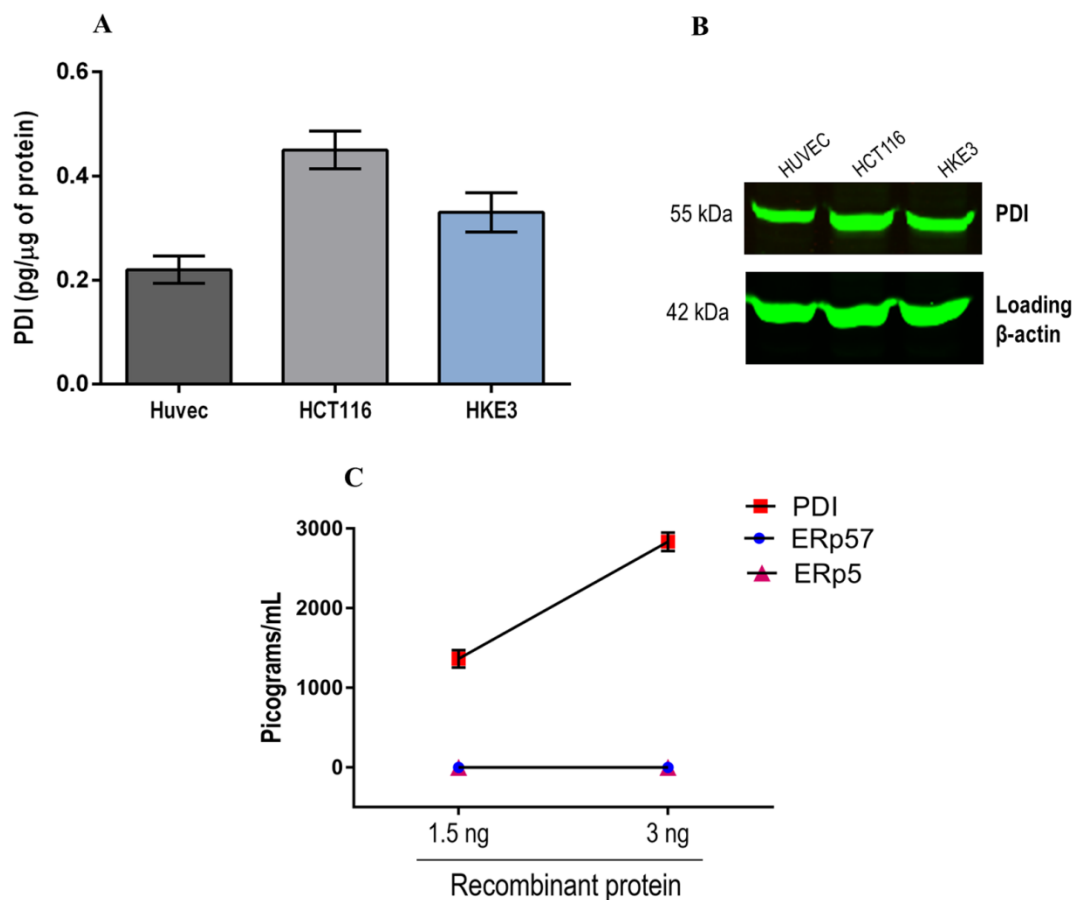


Figura 5 – Validação do ensaio de ELISA para detecção específica da PDI. A–B. Lisados de células humanas (HUVEC, HCT116 e HKE3) foram submetidos às seguintes análises (A) quantificação da concentração de PDI por ELISA (*Human P4HB Par Set, SinoBiological*) ($n= 4$; média \pm erro padrão) e (B) análise de *western blot* utilizando o anticorpo de captura do mesmo kit de ELISA ($n= 3$). C. Ensaio de reatividade cruzada com outros membros da família da PDI. As proteínas recombinantes PDI, ERp57 e ERp5 (1,5–3 ng/mL) foram quantificadas por ELISA. Dados representam média \pm erro padrão ($n= 4$).

O ensaio foi suficientemente capaz de detectar de forma específica um *pool* de PDI no plasma (como será discutido em detalhes abaixo, Figura 8). A detecção da PDI circulante foi adicionalmente confirmada por imunoprecipitação (Figura 6A e B). Além disso, investigamos se o *pool* de PDI presente no plasma está ativo utilizando um ensaio baseado na atividade redutase correspondente à clivagem da sonda di-eosina-GSSG e formação do produto reduzido EGSH, inibida pela rutina em amostras de plasma humano. O uso da rutina, um inibidor específico e bem validado na literatura para PDI (discutido na Introdução), confere especificidade ao ensaio, já que este mede atividade redutase total

incluindo várias proteínas capazes de reduzir dissulfetos. A rutina inibiu o *turnover* da di-eosina-GSSG em cerca de 70% e 45% nos plasmas ricos (PR-PDI) e pobres (PP-PDI) em PDI, respectivamente (Figura 7A); curvas representativas da cinética de atividade redutase mostrando os valores de fluorescência detectáveis no ensaio (RFU, unidade relativa de fluorescência) estão representadas na Figura 7B. Estes resultados evidenciam um *pool* plasmático ativo da PDI em indivíduos saudáveis. Uma abordagem conceitualmente análoga permitiu evidenciar a atividade da PDI plasmática em camundongos transgênicos com doença falciforme (81).

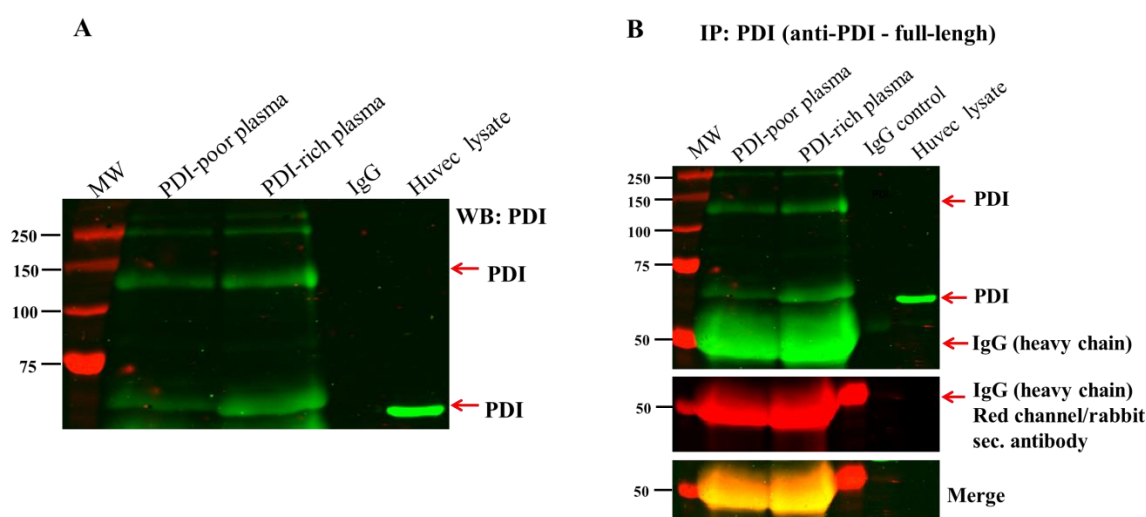


Figura 6 – PDI plasmática detectada por imunoprecipitação. **A.** Imunoprecipitação da PDI no plasma. Amostras de plasma pobre em plaquetas obtido de doadores com baixa ou alta concentração de PDI circulante foram diluídas em tampão de lise e imunoprecipitadas utilizando anticorpo anti-PDI (IgG de coelho). *Imunoblotting* foi realizado usando anticorpo anti-PDI (RL90) (IgG de camundongo). Lisado de HUVEC foi utilizado como controle positivo para peso molecular. ($n= 3$). **B.** *Imunoblottings* não cortados dos ensaios de imunoprecipitação mostram a ausência de reatividade cruzada entre a banda da PDI plasmática e a cadeia pesada da IgG utilizada para imunoprecipitação. Bandas da PDI são mostradas em verde (55 kDa e ~150 kDa). Controles mostram a co-localização em amarelo da cadeia pesada da IgG (50 kDa) em verde e vermelho. Canal verde (anti-camundongo), canal vermelho (anti-coelho).

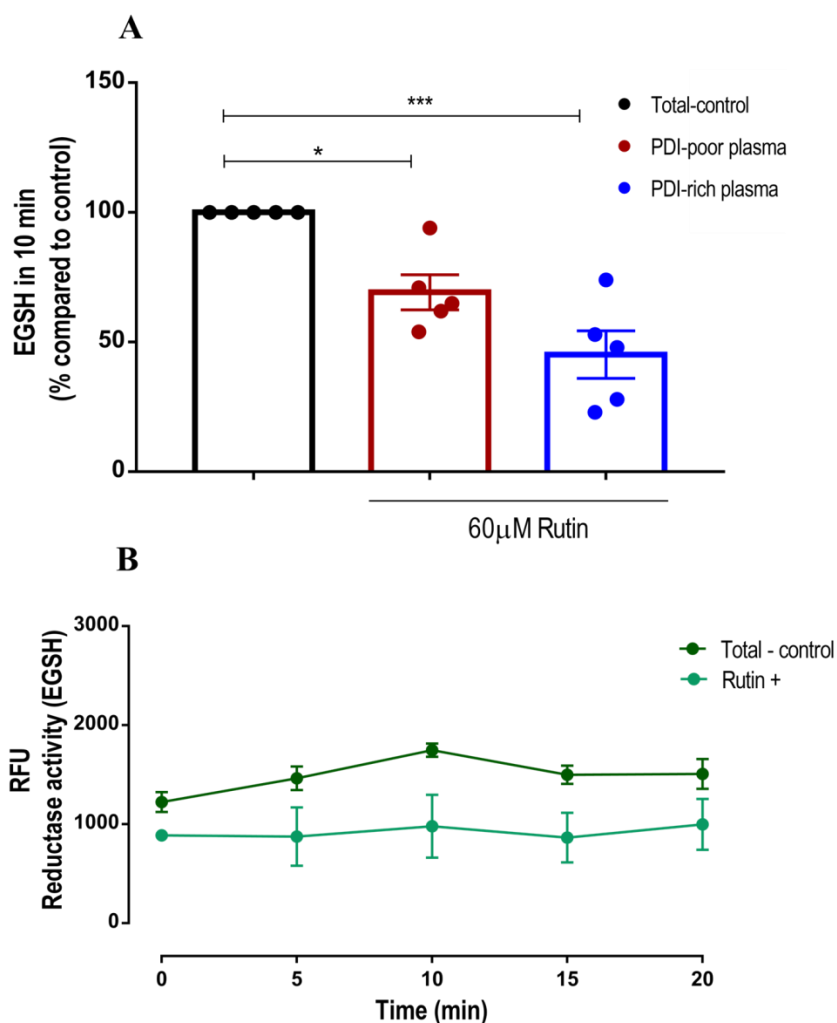


Figura 7 – Atividade redutase da PDI no plasma. **A.** A detecção da atividade redutase da PDI no plasma foi realizada usando a sonda di-eosina-GSSG. A formação do produto reduzido fluorescente (EGSH) foi medida em amostras de plasmas na presença ou na ausência de 60 μ M de rutina (inibidor da PDI) em fluorímetro de placa (emissão: 520 nm, excitação: 545 nm). Barras dos gráficos representam a porcentagem de clivagem da sonda di-eosina-GSSG em 10 min comparando a atividade redutase total (controle: 100%) em relação às amostras tratadas com rutina. O principal resultado neste caso refere-se à fração de atividade redutase (formação do EGSF) inibida pela rutina ($n=5$; média \pm erro padrão). *One-way ANOVA* e pós teste de *Tukey*. * $p<0,05$; *** $p<0,001$. **B.** Curva representativa da atividade redutase da PDI no plasma mostrada em (A). Formação do produto fluorescente foi acompanhada por 20 min. RFU, unidade relativa de fluorescência.

4.2. Concentração plasmática da PDI mostra alta variabilidade interindividual, porém baixa variabilidade intraindividual

Investigamos qual o perfil da PDI plasmática em indivíduos controles saudáveis, cujas características estão descritas na Tabela 1. A quantidade de PDI detectada por ELISA nas amostras de plasma analisadas foi baixa, em concentrações pM; a distribuição não seguiu os padrões de normalidade, como confirmado por testes específicos (Figura 8A e B). Investigamos também se a PDI estaria presente, e em que proporções, na fração de micropartículas (MP) do plasma, uma vez que a PDI e outras tiol isomerases estão presentes em MP isoladas a partir de CE e plaquetas humanas (68-70). Porém, a PDI associada a MP plasmáticas foi responsável apenas por uma pequena fração da concentração total de PDI no plasma em nossa população (Figura 9A).

Tabela 1. Característica da população de indivíduos saudáveis.

<i>n</i>	35	
Idade	34,2 ± 1,6	
Homens (%)	40	
Mulheres (%)	60	
Colesterol total, mg/dL	186,5 ± 5,1	
HDL, mg/dL	58,3 ± 2,8	
LDL, mg/dL	111,3 ± 5,1	
Não-HDL, mg/dL	128,7 ± 5,6	
Triglicerídeos, mg/dL	84,9 ± 8,2	
Contagem de plaquetas (×10 ⁹ /L)	263,6 ± 10,1	
Número de hemácias (×10 ¹² /L)	4,7 ± 0,1	
Número de leucócitos (×10 ⁹ /L)	6,7 ± 0,3	
Proteína C reativa, mg/L	2,6 ± 0,8	
Valores de PDI, pg/mL		
Mediana	330	Dados representam média ± erro padrão, exceto quando especificado. HDL: lipoproteína de alta densidade, LDL: lipoproteína de baixa densidade.
Média	539,4	
Percentil 25%	95	
Percentil 75%	918	

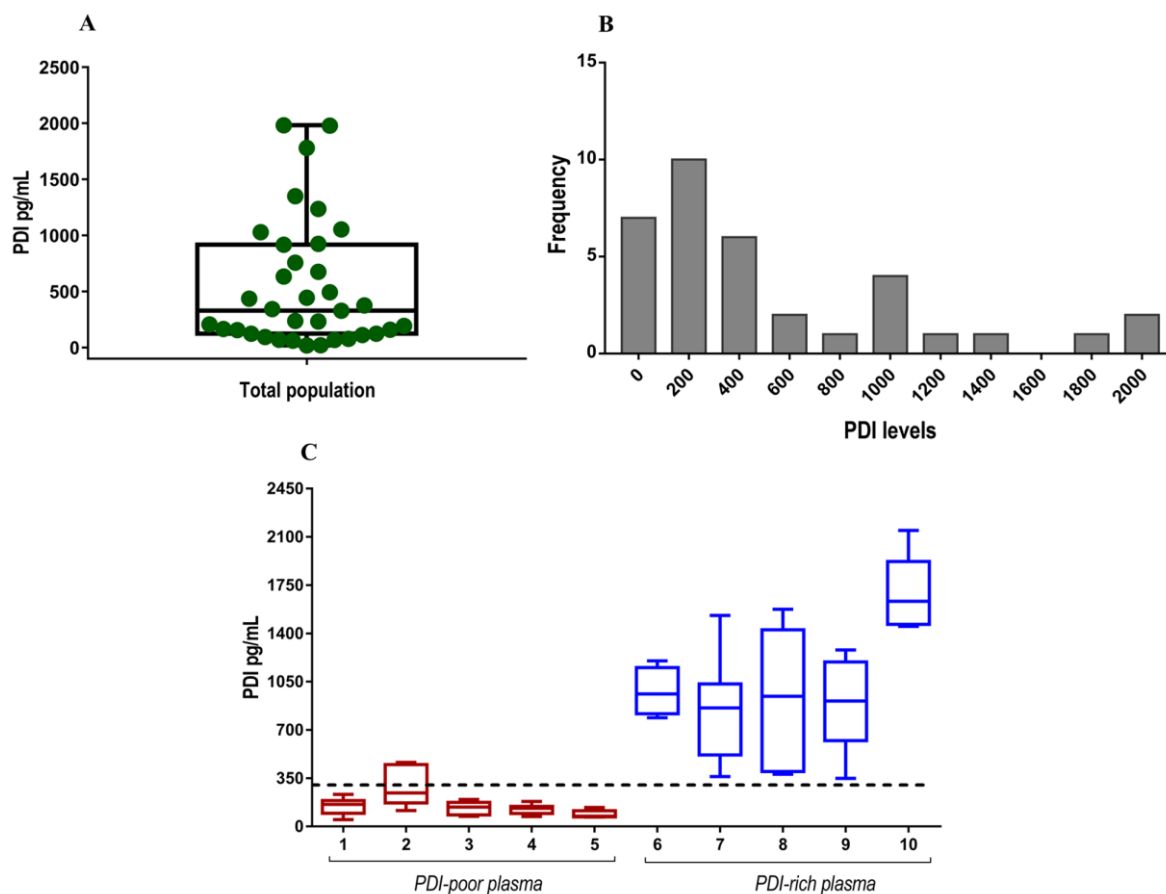


Figura 8 – Concentração plasmática da PDI em indivíduos saudáveis. Amostras de plasma foram coletadas a partir de doadores controles em diferentes ocasiões e os valores da PDI circulante determinados por ELISA. **A.** Distribuição da concentração plasmática da PDI na população. Box plot indica mediana e intervalos interquartis ($n=35$). **B.** Distribuição de frequência da concentração circulante da PDI mostrada em (A). **C.** Gráfico representativo da PDI plasmática de indivíduos saudáveis avaliados ao longo do tempo. Cada box plot representa um indivíduo e as diversas coletas de plasma realizadas em diferentes ocasiões ($n= 10-15$ amostras para cada indivíduo) como descrito nos Resultados. A linha pontilhada representa a mediana (330 pg/mL) da concentração plasmática da PDI na população mostrada em (A). Valores ≤ 330 pg/mL foram definidos como plasmas pobres em PDI [PP-PDI] e ≥ 330 pg/mL como plasmas ricos em PDI [PR-PDI]. Dados representam mediana e intervalos interquartis.

As concentrações plasmáticas da PDI mostraram alta variabilidade interindividual desde valores muito baixos/indetectáveis até valores superiores a 1000 pg/mL (~ 18 pM) (Figura 8C). Deste modo, consideramos que a concentração plasmática da PDI é bastante heterogênea nos indivíduos saudáveis. Como o valor mediano da população foi de 330 pg/mL (~ 6 pM) (Figura 8A) designamos valores ≤ 330 pg/mL como plasmas pobres [PP-PDI] em PDI (*PDI-poor plasma*), de maneira oposta valores > 330 pg/mL como plasmas

ricos em PDI [PR-PDI] (*PDI-rich plasma*). Importante, ao contrário da variabilidade interindividual, a variabilidade intraindividual foi muito pequena e pouco influenciada por fatores externos aparentes (Figura 8C). As coletas de plasma foram repetidas em diferentes ocasiões e/ou condições (10–15 vezes espaçadas entre dias e semanas durante 10–15 meses), utilizando os mesmos indivíduos e os resultados mostraram valores muito similares e pouco variáveis na concentração da PDI no plasma (Figura 8C). Para investigar esta variabilidade intraindividual, avaliamos, em uma pequena coorte de 5 indivíduos, se a concentração plasmática da PDI variava ao longo do dia. Amostras de plasma foram coletadas em 3 períodos diferentes durante o dia (período aproximado de 9h). Primeira coleta, em jejum (8h), 2ª coleta, após o almoço (13h) e a 3ª coleta, final da tarde (17h). Os resultados mostram que a variabilidade intraindividual dos valores plasmáticos da PDI é baixa e se mantêm muito parecida em diferentes horas do dia (Figura 9B).

Outro aspecto importante foi a análise da possível influência de variáveis que refletem fatores de risco tradicionais cardiovascular e inflamatório nos valores de PDI no plasma. Nossos resultados não mostraram correlação significativa entre os valores plasmáticos da PDI com idade, valores de triglicerídeos, lipoproteína de alta densidade (HDL), proteína C-reativa, contagem de leucócitos, hemácias e plaquetas. Houve uma correlação direta não robusta com os valores de colesterol total e LDL ($p < 0,05$) (Figura 10).

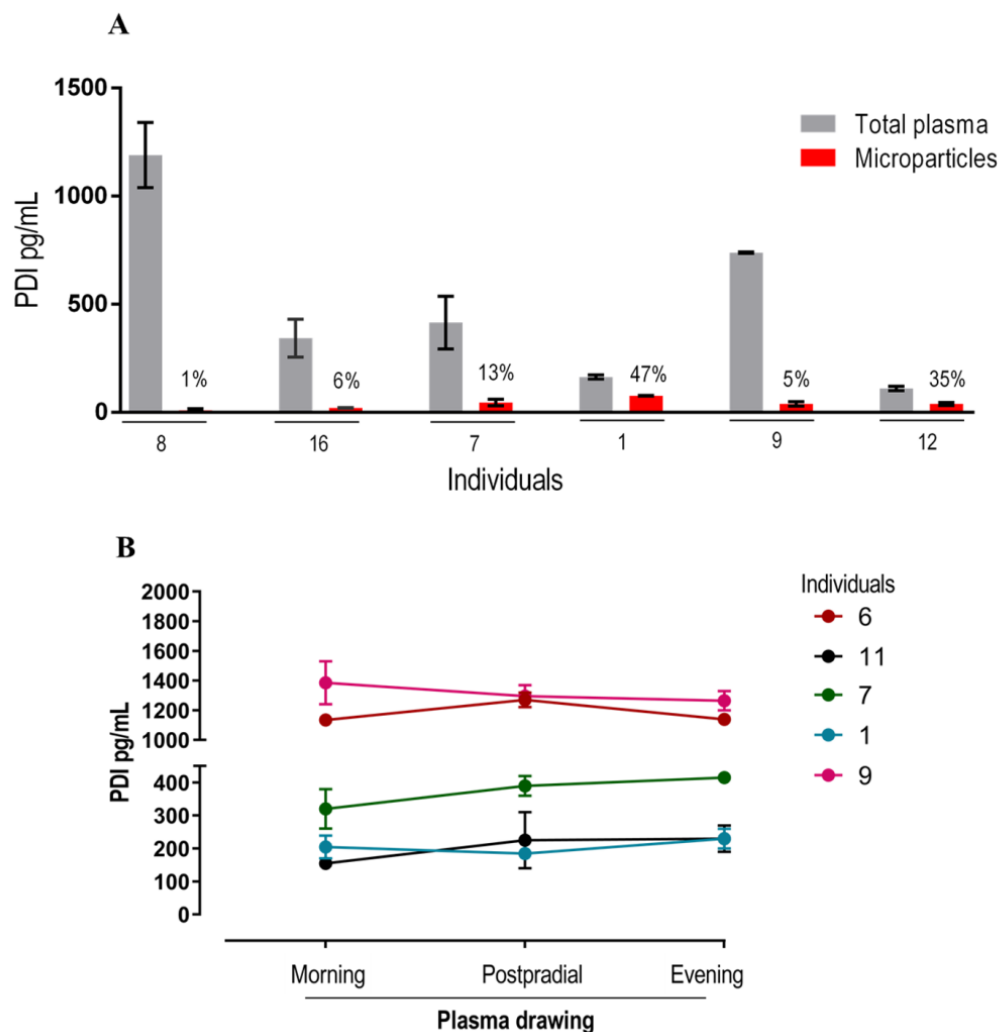


Figura 9 – Características da PDI no plasma. A. Detecção da PDI em micropartículas plasmáticas. A fração de micropartículas foi obtida a partir dos plasmas de indivíduos controles isoladas através de sucessivas ultracentrifugações e posterior lise. As concentrações da PDI foram detectadas por ELISA. As barras representam média \pm erro padrão ($n=3$). A proporção de PDI presente em micropartículas em relação à concentração plasmática total está representada em porcentagem no gráfico. **B.** Variabilidade intraindividual da concentração plasmática da PDI ao longo do dia. Amostras de plasma foram coletadas em 3 períodos diferentes durante o dia. Primeira coleta, em jejum (8h), 2^o coleta, após o almoço (13h) e a 3^o coleta, final da tarde (17h). A concentração da PDI foi determinada por ELISA. As barras representam média \pm erro padrão ($n=3$).

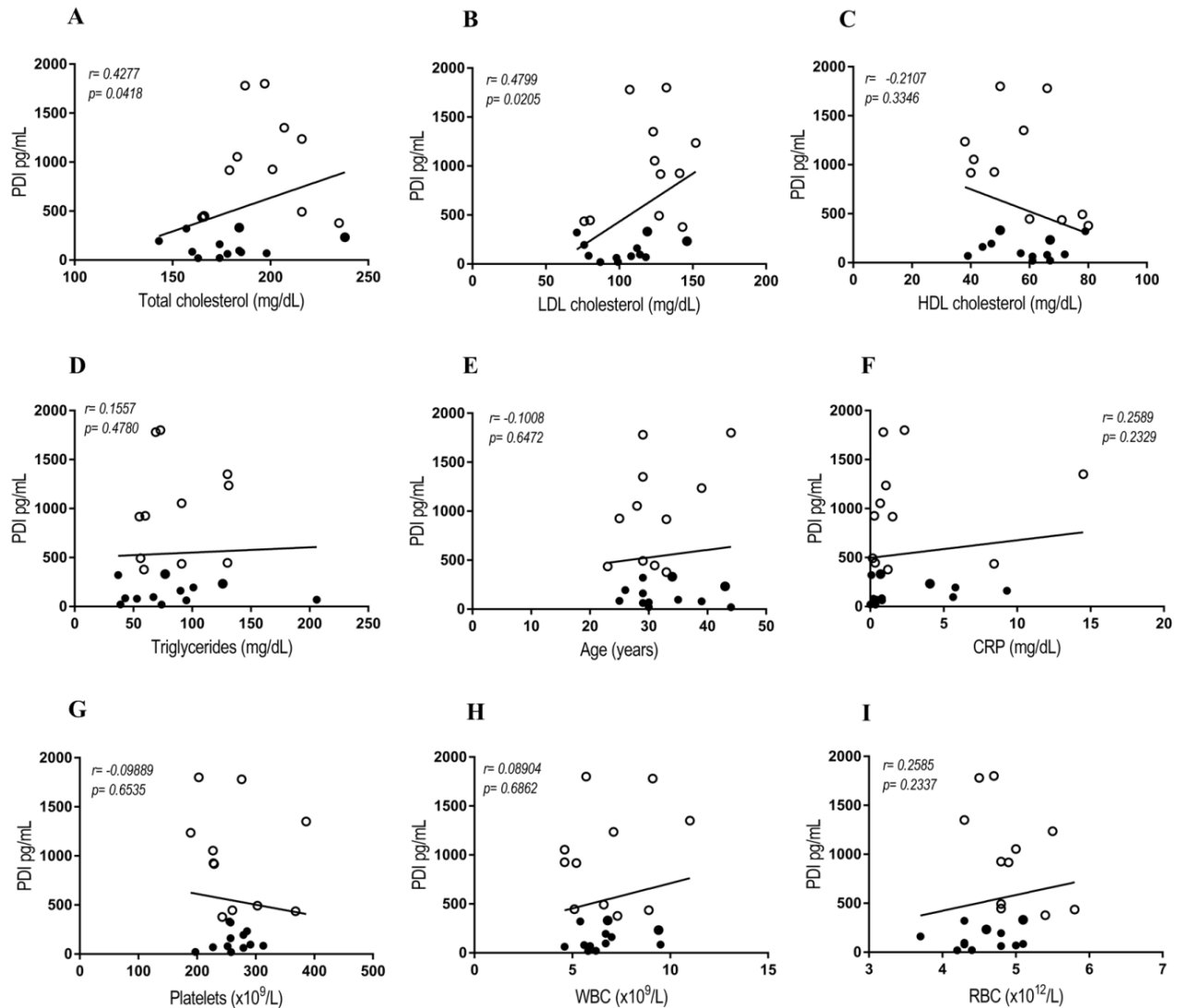


Figura 10 – Correlação entre a concentração plasmática da PDI e diferentes variáveis individuais. A. Colesterol total. B. Lipoproteína de baixa densidade (LDL). C. Lipoproteína de alta densidade (HDL). D. Triglicérides. E. Idade. F. Proteína C reativa. G. Número de plaquetas. H. Número de leucócitos. I. Número de eritrócitos. Todas as variáveis foram representadas em função da concentração plasmática da PDI. Círculos fechados e abertos representam respectivamente, indivíduos com concentrações baixas e altas PDI no plasma ($n=23$). Coeficiente de correlação (r) e valor de p foram calculados de acordo com o método de *Spearman*; $p < 0,05$ foi considerado significativo.

4.3. Estado redox do pool de PDI no plasma

Avaliamos o estado redox da PDI no plasma, uma vez que este é essencial para suas funções (17). A PDI exibe um *pool* de estados redox distintos: totalmente/parcialmente reduzido, totalmente/parcialmente oxidado a dissulfetos intramoleculares ou oxidado a dissulfetos intermoleculares (p. ex., S-glutationilado) ou S-nitrosilado. Investigamos o estado redox da PDI plasmática por meio da utilização de sondas biotiniladas (descrito em detalhes nos Métodos). Os resultados descritos na Figura 11A mostram que a maior parte (60–80%) do *pool* de PDI no plasma está reduzido. Não houve diferença no estado redox da PDI entre as amostras de PP-PDI vs. PR-PDI. A Figura 11B mostra os valores de densidade óptica (OD) lidos em espectrofotômetro para cada condição avaliada. Os valores obtidos pela marcação com a sonda Sulfo-NHS-biotina referem-se à quantidade total de PDI medida no ensaio, uma vez que esta molécula marca as aminas primárias de proteínas, fornecendo uma ferramenta capaz de biotinar a totalidade (ou quase) de proteínas nas amostras avaliadas. Os valores obtidos pela marcação com MPB referem-se à fração de PDI reduzida, uma vez que a sonda se liga apenas aos tióis livres. É importante mencionar que utilizamos todas as sondas em excesso (4 mM) para garantir a biotinação máxima em cada amostra. Em todos os ensaios amostras não biotiniladas foram avaliadas como controles (*No labeling*). Uma amostra obtida a partir de um *pool* de plasmas foi utilizada como um controle padrão interno em todos os ensaios para analisar variações na biotinação de proteínas plasmáticas com as sondas utilizadas. Como mostrado na Figura 11C, a variabilidade na eficiência de biotinação foi pequena entre os ensaios.

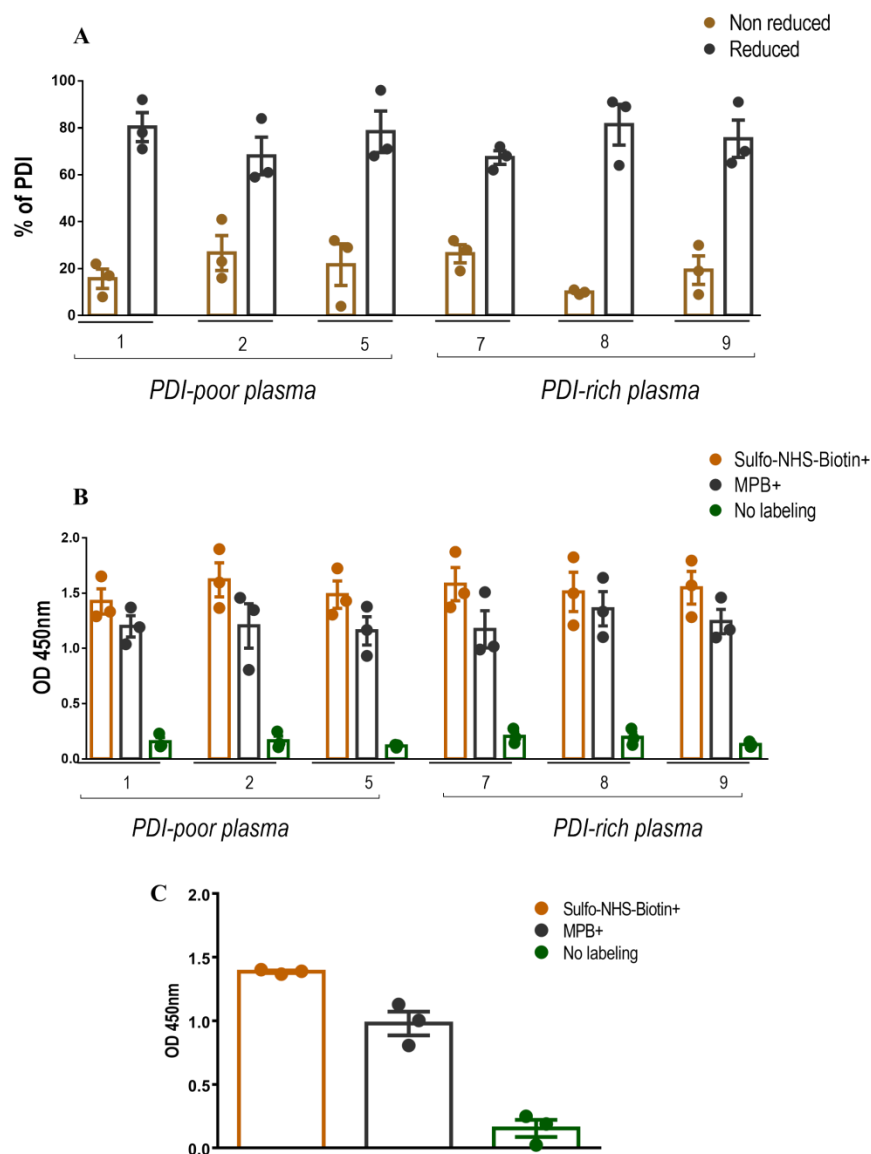


Figura 11 – Detecção do estado redox da PDI no plasma humano. A–C. Amostras de plasma foram biotiniladas utilizando 4 mM de EZ-Link Sulfo-NHS-Biotina (marcação de aminas primárias) e 4 mM de MPB (marcação de tióis livres) em seguida precipitadas com acetona (remoção de produtos não ligados). Os *pellets* de proteínas foram ressuspensos em PBS-tween e analisados por ELISA como descrito em detalhes nos Métodos. **A.** Porcentagem de PDI reduzida e não-reduzida no plasma de indivíduos com baixa e alta concentração de PDI. A porcentagem foi resultante da comparação entre a PDI marcada com MPB (fração reduzida) vs. EZ-link Sulfo-NHS-Biotina (fração total). Números representam diferentes indivíduos ($n=3$; média \pm erro padrão). **B.** Valores de absorvâncias (densidade óptica, OD) medidas em 450 nm para cada uma das condições mencionadas em (A). Os mesmos valores foram utilizados para os cálculos de porcentagem mostrados em (A). Amostras de plasma foram submetidas aos mesmos procedimentos, em paralelo, na ausência das sondas biotiniladas. (controle não marcado). **C.** Um *pool* de plasmas isolados a partir de 3 doadores foi utilizado como controle padrão interno em todos os ensaios para analisar variações na biotinilação de proteínas plasmáticas com MPB ou Sulfo-NHS-Biotina entre os ensaios. As barras representam os valores de OD para cada condição ($n=3$; média \pm erro padrão).

4.4. Plasmas com baixas vs. altas concentrações de PDI revelam distintos perfis de composição de proteínas

Diante da baixa variabilidade intraindividual, apesar dos valores interindividuais amplamente distintos, hipotetizamos que a concentração de PDI poderia se comportar como uma janela para revelar padrões individuais de perfis proteicos plasmáticos. Para investigar este aspecto, foi realizada uma análise proteômica quantitativa comparando os PP-PDI e PR-PDI de indivíduos saudáveis em nossa população de estudo. As amostras foram depletadas das 14 principais proteínas com grande abundância no plasma, pois estas tendem a mascarar proteínas de baixa abundância em estudos proteômicos devido ao seu intervalo de concentração grande e dinâmico. O sistema MARS-14 (Sistema de remoção de múltipla afinidade) foi utilizado para este fim, permitindo o fracionamento dos plasmas em duas partes: proteínas mais abundantes (primariamente constitutivas e "housekeepings") e proteínas menos abundantes (que contém proteínas associadas à sinalização e outros processos celulares). Após depleção, as proteínas de baixa abundância foram analisadas por proteômica baseada em espectrometria de massa, resultando na identificação de 1788 proteínas no proteoma total. A análise quantitativa revelou 125 proteínas diferencialmente reguladas, sendo 65 proteínas mais expressas nos PP-PDI e 60 proteínas mais expressas nos PR-PDI (Anexos B e C).

Para investigar em cada tipo de plasma possíveis perfis proteômicos associados a processos específicos, foi utilizado o banco de dados do *software Metacore*. De acordo com os resultados da análise de enriquecimento de vias canônicas, o subconjunto de proteínas diferencialmente expressas nos PP-PDI foi associado preferencialmente a imunoinflamação, coagulação e interações plaquetárias; 50% das vias e 70% dos processos biológicos incluíram coagulação sanguínea e resposta imune. Os principais exemplos destas proteínas incluem: os fatores de coagulação XI e XII e cininogênio 1, ambos envolvidos no início da via intrínseca da cascata de coagulação; proteínas associadas a interações com plasminogênio e fibrinogênio; proteínas envolvidas em interações entre células e plaquetas, como a glicoproteína rica em histidina (HRG) (82, 83), proteínas relacionadas ao sistema imune como a glicoproteína rica em leucina alfa-2 (84) e CD14 (85). Além disso, diversas proteínas das vias do complemento foram preferencialmente identificadas nos PP-PDI.

Todas as vias e processos biológicos, bem como as ontologias gênicas (*gene ontology, GO*) identificadas nas redes biológicas estão apresentados nas Figuras 12 (painel esquerdo) e Figura 13A. De maneira oposta, o conjunto de proteínas diferencialmente expressas nos PR-PDI foi associado principalmente a proteínas relacionadas com adesão celular (p. ex.: caderina-5), citoesqueleto (p. ex.: miosina-8), ciclo celular (p. ex.: proteína do tipo 1 semelhante à MAD-2). Todas as vias e processos biológicos, bem como as ontologias gênicas (*gene ontology, GO*) identificadas nas redes biológicas estão apresentados nas Figuras 12 (painel direito) e Figura 13B. Estes resultados sugerem que os valores de PDI podem discriminar distintos processos biológicos e ontologias gênicas associados a perfis proteômicos específicos nos plasmas de indivíduos saudáveis, sem outras diferenças óbvias em seus indicadores convencionais de risco cardiovascular.

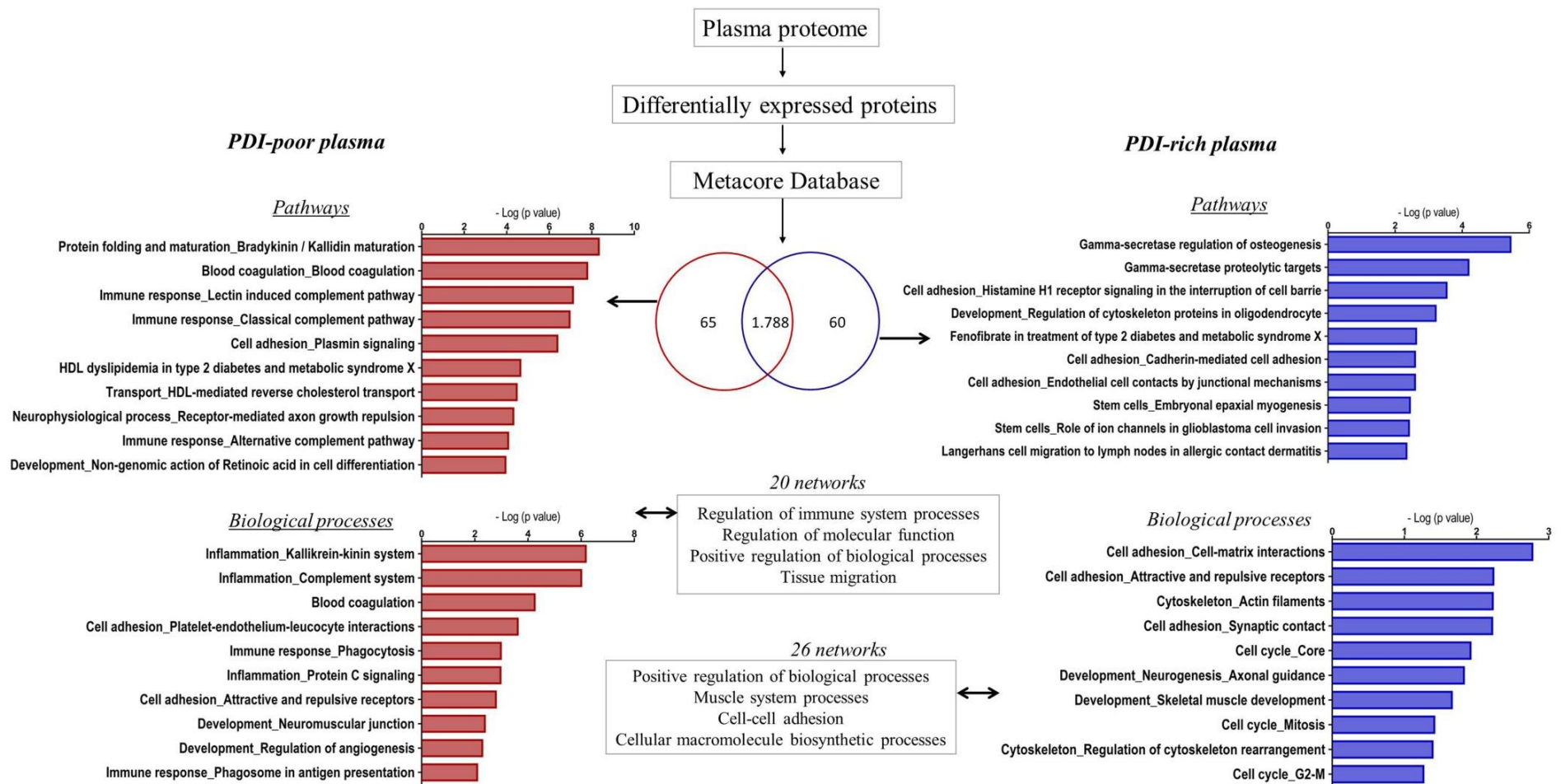


Figura 12 – Proteoma total e análise de enriquecimento de vias e processos biológicos de proteínas diferencialmente expressas nos PP-PDI e PR-PDI. Amostras de plasma de indivíduos com baixa ($n=3$) e alta ($n=3$) concentração circulante de PDI foram submetidas à análise de espectrometria de massa (*shotgun*) para a identificação de proteínas diferencialmente expressas em cada condição. Foram identificadas 65 e 60 proteínas com expressão aumentada nos PP-PDI e PR-PDI, respectivamente. Análises adicionais foram realizadas utilizando o *software Metacore*, incluindo identificação de vias e processos biológicos associados a cada lista de proteínas ($p<0,05$).

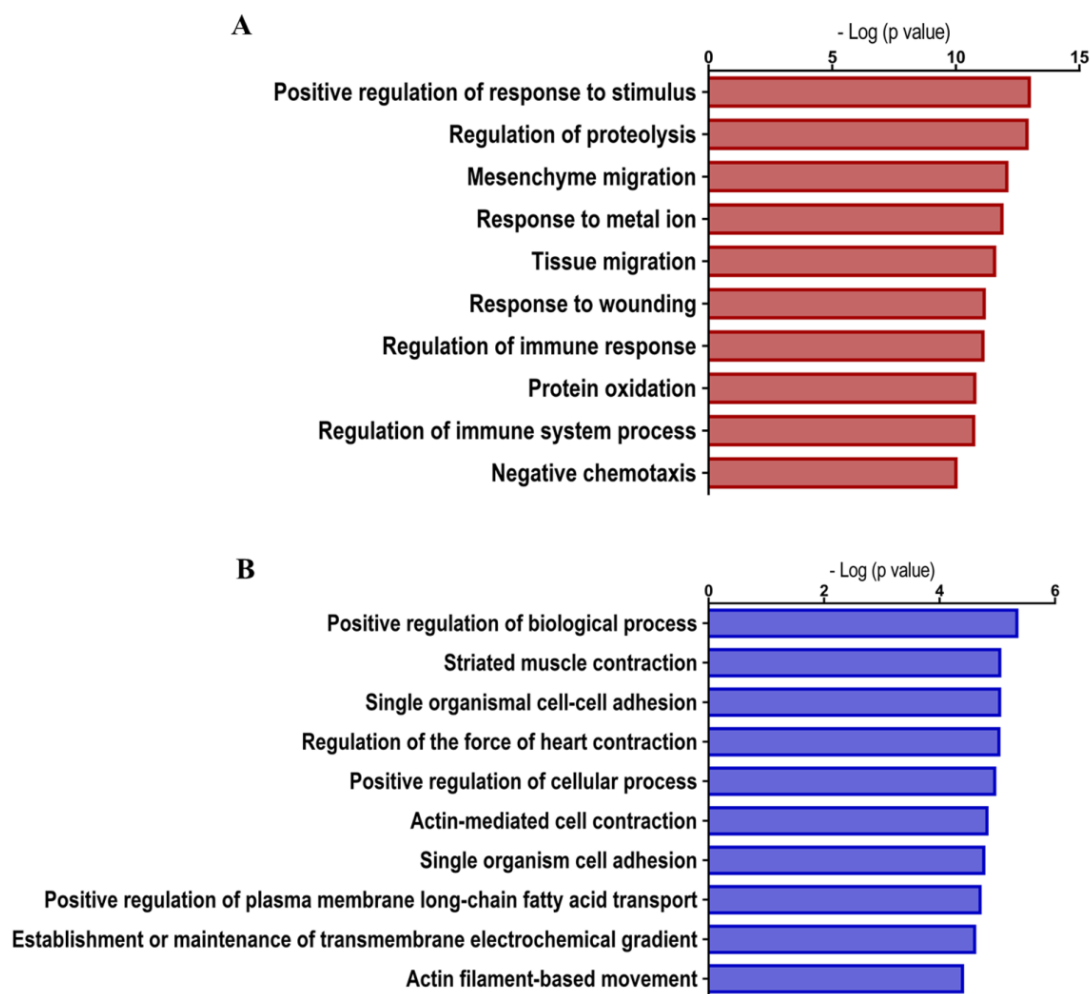


Figura 13 – Processos de ontologia genica (*gene ontology, GO*) das proteínas identificadas no proteoma total do plasma. As proteínas de maior expressão entre os indivíduos com PP-PDI e PR-PDI foram identificadas por espectrometria de massa e analisadas pelo *software Metacore*. Os principais processos GO ($p < 0,05$) foram representados nos gráficos a seguir. A. Plasmas pobres em PDI (PP-PDI) ($n=3$). B. Plasmas ricos em PDI (PR-PDI) ($n=3$).

4.5. Perfis proteômicos plasmáticos discriminados pela PDI não se correlacionaram com padrão de agregação plaquetária

Considerando que a PDI é essencial para a agregação de plaquetas (86-89) e exerce papel crítico no crescimento do trombo vascular após lesão (39, 50, 66, 90, 91), questionamos a seguir se as diferenças observadas nos valores plasmáticos da PDI se associavam a diferentes perfis de atividade plaquetária. Para investigar esta questão, ensaios de agregação plaquetária em plasma rico em plaquetas (PRP) foram realizados em

PP-PDI vs. PR-PDI PDI, estimulados por 5 min com ADP (10 μ M), colágeno (5 μ g/mL) e ionóforo de cálcio – A23187 (20,5 μ M). As respostas de agregação plaquetária a distintos agonistas não mostraram diferenças entre os PP-PDI vs. PR-PDI. O estímulo de cada um dos agonistas induziu a mesma intensidade de agregação plaquetária (em média cerca de 80%) (Figura 14). Investigamos adicionalmente a agregação de plaquetas no sangue total e de novo, nenhuma diferença foi observada comparando indivíduos com menor ou maior quantidade de PDI circulante (Figura 15). Neste caso, apenas o colágeno foi utilizado como agonista e o controle sem estímulo agregador foi obtido por meio da incubação das amostras com solução salina.

Como a PDI é secretada após ativação plaquetária investigamos a seguir, a quantidade de PDI solúvel no plasma após agregação das plaquetas. Ao final de 5 min de agregação, os PRPs foram centrifugados e os plasmas pobres em plaquetas resultantes foram submetidos à quantificação de PDI por ELISA. Curiosamente, os valores plasmáticos da PDI foram diminuídos após agregação plaquetária na maioria das amostras, de maneira independente do agonista utilizado. No entanto, poucas exceções foram observadas (Figura 16). As amostras controles foram geradas na ausência dos agonistas. Ambos os efeitos observados nos experimentos de atividade plaquetária não estavam relacionados a concentração circulante da PDI ou com a intensidade de agregação das plaquetas.

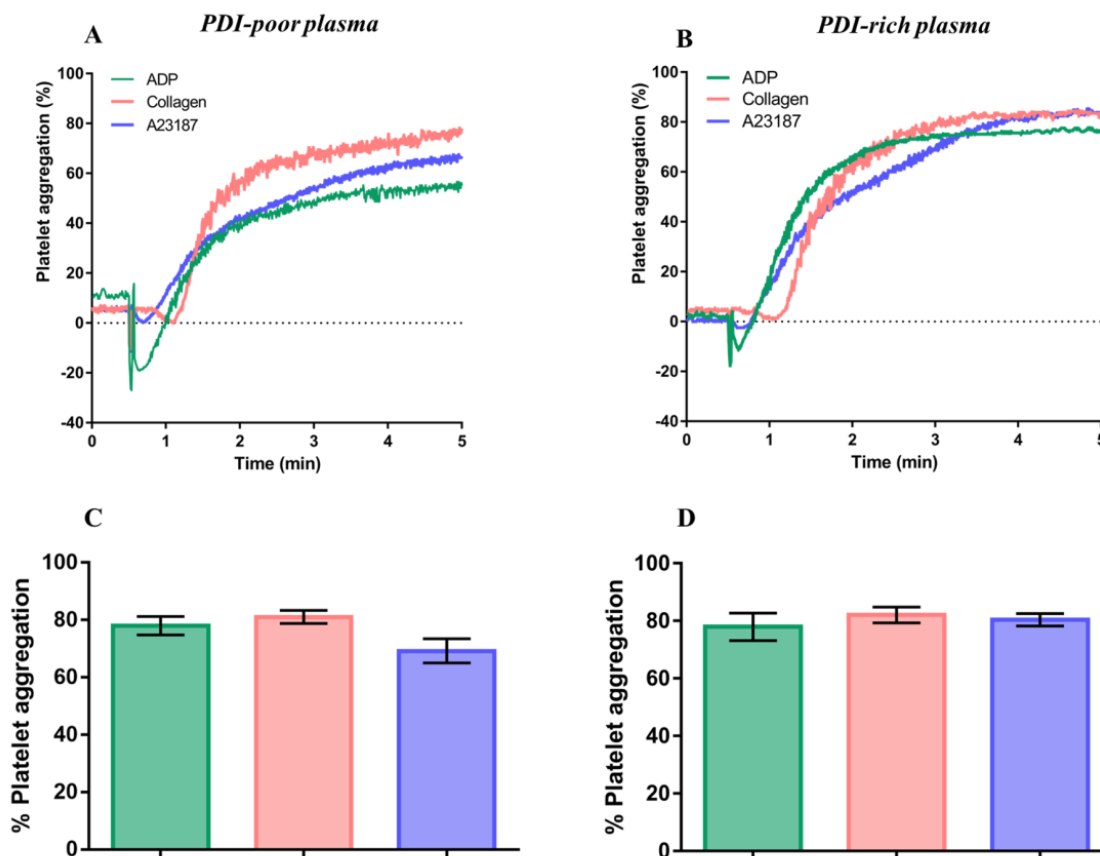


Figura 14 – Agregação plaquetária em plasma rico em plaquetas. Os experimentos foram realizados em plasma rico em plaquetas (PRP) isolado de indivíduos com baixas ou altas concentrações de PDI. O PRP ($2-3 \times 10^8$ plaquetas mL^{-1}) foi estimulado com $10 \mu\text{M}$ de ADP, $5 \mu\text{g/mL}$ de colágeno e $20,5 \mu\text{M}$ de A23187 (ionóforo cálcio) por 5 min a 37°C . **A.** Curvas de agregação plaquetária representativa para os PP-PDI. **B.** Curvas de agregação plaquetária representativa para os PR-PDI. **C.** Extensão máxima de agregação mostrada em (A). **D.** Extensão máxima de agregação mostrada em (B). Todos os dados representam média \pm erro padrão de 6–7 experimentos independentes. *One-way ANOVA* e pós teste de *Tukey*. Nenhuma diferença foi detectada na extensão de agregação utilizando diferentes agonistas nos PP-PDI e PR-PDI.

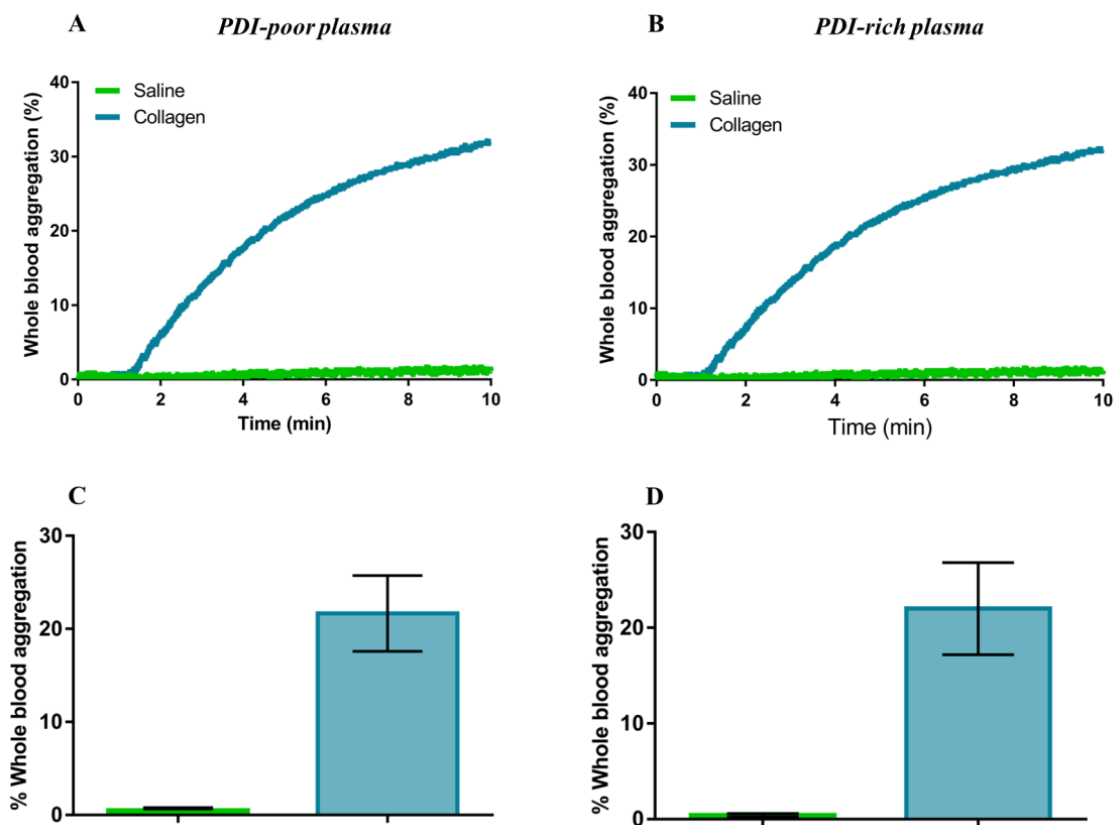


Figura 15 – Agregação plaquetária em sangue total. Os experimentos foram realizados em sangue total isolado de indivíduos com baixa e alta concentração de PDI. O sangue total foi diluído em solução salina ($2-3 \times 10^8$ plaquetas mL^{-1}) e estimulado com $25 \mu\text{g/mL}$ de colágeno ou incubado com solução salina (controle negativo) por 10 min a 37°C . **A.** Curva de agregação plaquetária representativa para os PP-PDI. **B.** Curva de agregação plaquetária representativa para os PR-PDI. **C.** Extensão máxima de agregação mostrada em (A) ($n=6$; média \pm erro padrão). **D.** Extensão máxima de agregação mostrada em (B) ($n=7$; média \pm erro padrão). *One-way ANOVA* e pós teste de *Tukey*. Nenhuma diferença foi detectada na extensão de agregação entre os PP-PDI e PR-PDI.

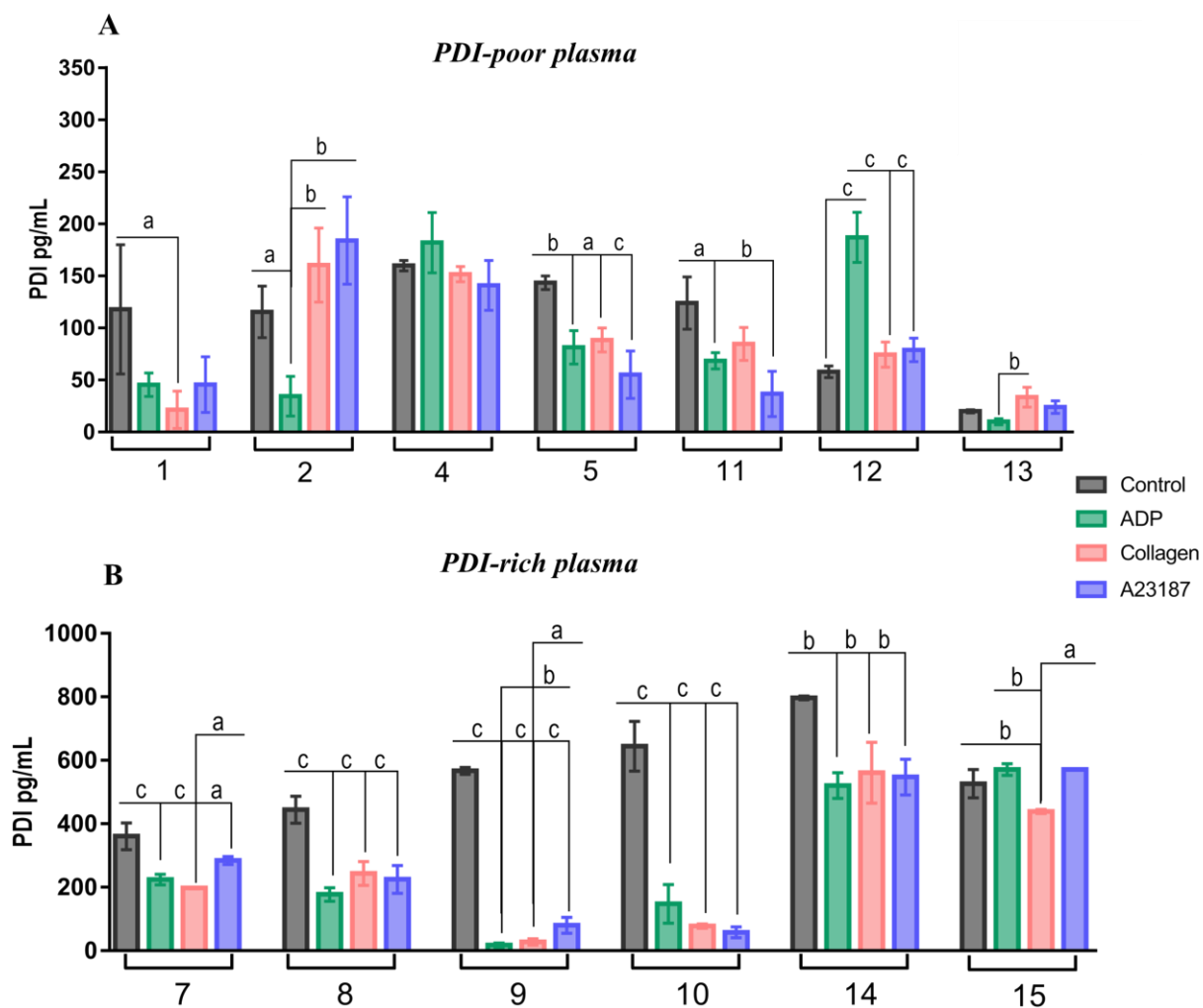


Figura 16 – Detecção da PDI solúvel nos plasmas após agregação plaquetária. A PDI solúvel foi medida por ELISA nos sobrenadantes de plasmas ricos em plaquetas (PRP) em repouso (controle) e estimulados com 10 μ M de ADP, 5 μ g/mL de colágeno e 20,5 μ M de A23187 (ionóforo cálcio) por 5 min a 37°C como descrito nos Métodos. **A.** Sobrenadantes dos PRPs (plasmas pobres em plaquetas) obtidos de indivíduos com PP-PDI. **B.** Sobrenadantes dos PRPs (plasmas pobres em plaquetas) obtidos de indivíduos com PR-PDI. Os dados representam média \pm erro padrão de 6–7 experimentos independentes. ^a $p < 0,05$; ^b $p < 0,005$; ^c $p < 0,0005$ (*One-way ANOVA* e pós teste de *Tukey*).

4.6. Diferentes concentrações de PDI no plasma recapitularam perfis opostos de expressão gênica e secreção em células endoteliais

Diversos grupos, incluindo nosso laboratório, mostraram anteriormente que CE podem secretar PDI (39), mesmo no estado basal e ausência de ativação (28). Além disso, a PDI extracelular se associa a uma série de efeitos envolvendo CE, particularmente em relação à secreção de PDI após ativação de integrinas (92), iniciação de trombose (59) e adesão de neutrófilos durante inflamação vascular (93). Desta forma, as diferentes assinaturas proteômicas reveladas pelos distintos valores plasmáticos da PDI podem interagir intimamente com fenótipo e função endotelial. Assim, avaliamos a seguir os efeitos da incubação de PP-PDI vs. PR-PDI adicionados exogenamente a CE humanas (linhagem de cordão umbilical humano HUVEC) cultivadas.

HUVEC foram incubadas por 24h em meio de cultura com 5% soro fetal bovino padrão (FBS) ou em meio no qual FBS foi substituído por 5% de um *pool* de plasmas coletados de indivíduos com PP-PDI ou PR-PDI. Os padrões de expressão gênica e secreção de proteínas foram avaliados em cada condição. As alterações na expressão de mRNA foram analisadas pela técnica de PCR utilizando um painel cobrindo 84 genes pré-especificados (*PCR array*) envolvidos em processos celulares que incluíram coagulação sanguínea, adesão celular, transporte e metabolismo de lipídeos, inflamação e quimiotaxia, crescimento e proliferação celular, bem como apoptose (Anexo D). CE expostas aos plasmas mostraram alterações fenotípicas consistentes com sua ativação, uma vez que a maioria das células adquiriram morfologia semelhante à de fibroblastos, perdendo seu fenótipo normal semelhante a paralelepípedo (*cobblestone-like*) (Figura 17). Referimos à ativação de CE como mudança em seu perfil sintético basal para um estado ativado, que se caracteriza como pró-trombótico, pró-proliferativo e vasoconstritor (94, 95).

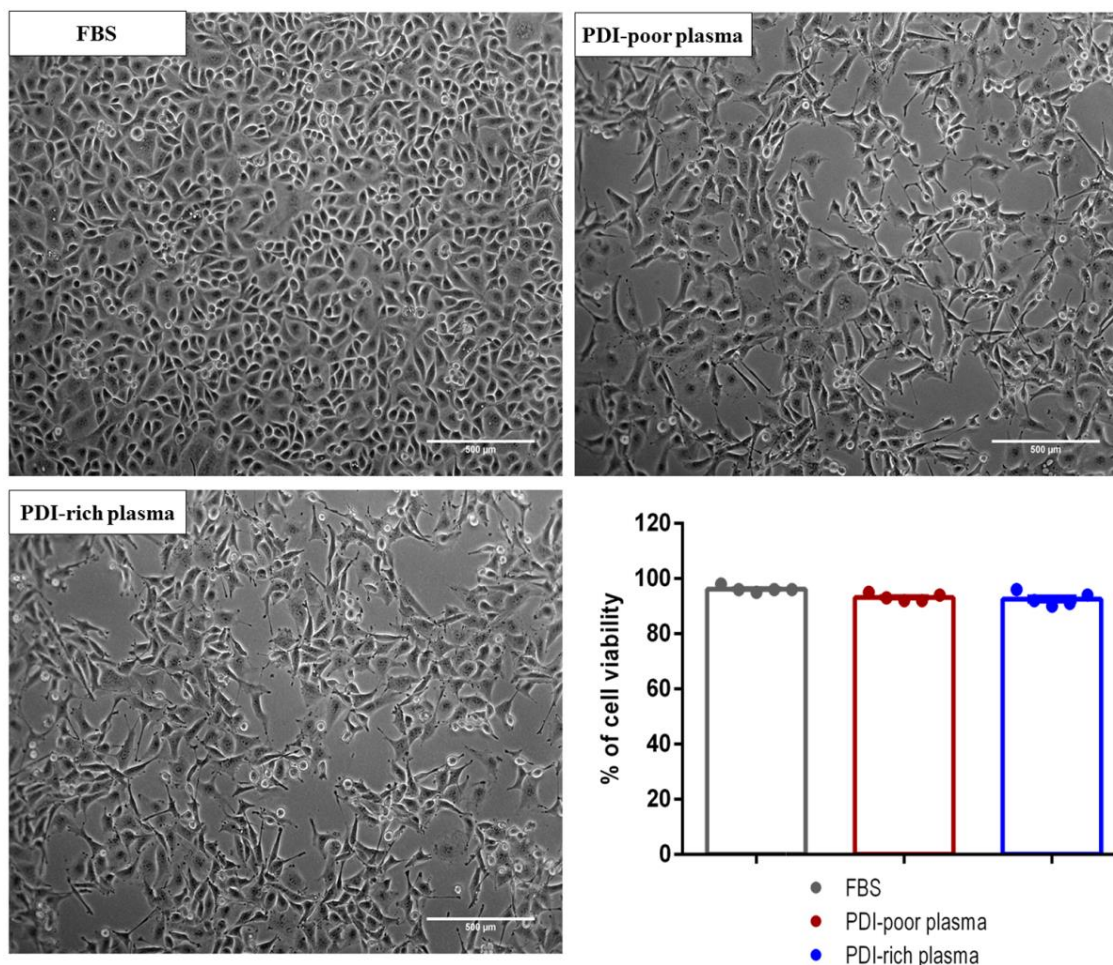
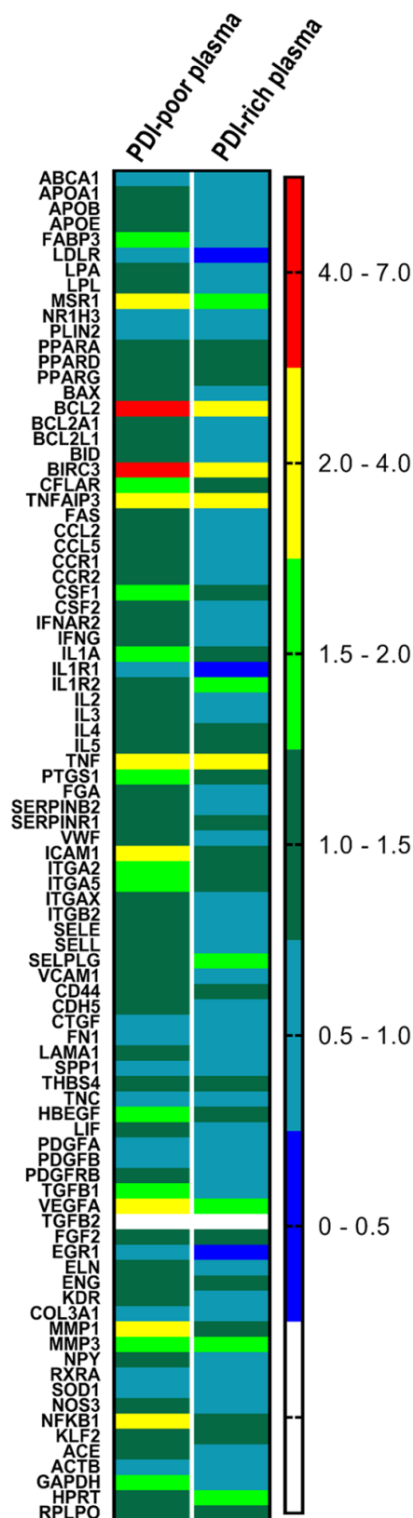


Figura 17 – Viabilidade de células endoteliais (HUVEC) incubadas com PP-PDI e PR-PDI. A viabilidade celular foi medida por ensaio de exclusão do corante azul de *Tripan*. HUVEC foram incubadas por 24h em meio RPMI no qual o soro fetal bovino (FBS) foi substituído por 5% de um pool de PP-PDI ou PR-PDI. Após este período as células foram lavadas, descoladas e submetidas à coloração com o corante. Fotomicrografias representativas. As barras representam média \pm erro padrão ($n= 5$).

Embora as mudanças morfológicas fossem indistinguíveis entre CE incubadas com PP-PDI ou PR-PDI, houve diferenças notáveis na expressão dos genes para cada tipo de plasma vs. Controle-FBS, sugerindo padrões distintos de ativação endotelial, como demonstrado na Figura 18. Análise na mudança na expressão de mRNAs (aqui definida como mudança ≥ 2 vezes) mostrou que 48% dos genes foram simultaneamente regulados positivamente (ou seja, tiveram expressão aumentada) quando CE foram incubadas com PP-PDI e regulados negativamente (ou seja, tiveram expressão diminuída) quando CE foram incubadas com PR-PDI, ambos após 24h de incubação (Figura 19). Os principais

exemplos incluíram integrinas envolvidas na adesão de leucócitos-EC: integrina ITGAL/ITGB2 (ITGB2), $\alpha X\beta 2$ (ITGAX), molécula de adesão intracelular-1 (ICAM1), selectinas E (SELE) e L (SELL), bem como os genes relacionados à coagulação sanguínea: fator de von Willebrand (VWF), cadeia alfa do fibrinogênio (FGA) e inibidores de fibrinólise como inibidor do ativador de plasminogênio 1 e 2 (SERPINE1, SERPINB2, respectivamente). Um padrão idêntico foi encontrado para 10 dos 17 genes envolvidos em inflamação e quimiotaxia, enquanto 6 dos demais genes tiveram sua expressão aumentada após a incubação tanto com PP-PDI como PR-PDI. A expressão do receptor da interleucina-1 (IL1R) foi regulada negativamente nas mesmas condições (Figuras 19 e 20). Entre 14 genes relacionados a transporte e metabolismo de lipídeos, 6 também tiveram sua expressão aumentada após exposição aos PP-PDI e ao mesmo tempo expressão diminuída quando expostos aos PR-PDI, enquanto 8 mostraram comportamentos diversos (Figuras 19 e 20).

Em relação aos genes de crescimento e proliferação celular, não observamos correlação clara com a exposição de CE aos diferentes plasmas: o receptor de PDGF (PDGFRB) e o TGF- $\beta 1$ tiveram expressão diminuída quando CE foram incubadas com os PP-PDI e aumentada quando incubadas com os PR-PDI; o fator de transformação de crescimento β -2 (TGFB2) e o fator de crescimento endotelial vascular (VEGFA) foram regulados positivamente em ambos os casos; os genes de fatores de crescimento derivados de plaquetas (PDGFB e A) e de resposta rápida ao crescimento (EGR1) foram regulados negativamente em ambos os casos. Entre os 9 genes relacionados à apoptose, 5 tiveram sua expressão aumentada quando as CE foram incubadas com PP-PDI e simultaneamente diminuída quando incubadas com PR-PDI (FAS, BCL2L1, BCL2A1, BID, BAX), enquanto 4 foram aumentadas em ambos os casos (Figuras 19 e 20). Os genes anti-apoptóticos BCL2 e BIRC3 tiveram a maior mudança de expressão dos mRNAs analisados em comparação com o controle, com suas expressões acentuadas mediante incubação das células com PP-PDI (Figuras 19 e 20). É válido ressaltar que BCL2 e BIRC3 regulam não apenas caspases e apoptose, mas também os processos inflamatórios de sinalização e imunidade (96, 97). Apesar da ativação de genes apoptóticos, em nenhum dos casos houve perda detectável da viabilidade celular, pois a maioria das células permaneceram viáveis



após 24h de incubação com os PP-PDI ou PR-PDI, assim como os controles incubados com FBS (Figura 17).

Os resultados descritos acima sugerem que a incubação de EC com plasmas de indivíduos com baixa ou alta concentração de PDI parecem recapitular um padrão de alterações na expressão gênica endotelial similar e correspondente às assinaturas proteômicas plasmáticas identificados nestes indivíduos.

Figura 18 – Heat map do mRNA de genes envolvidos em diversos eventos celulares após a exposição de células endoteliais a PP-PDI e PR-PDI. HUVEC foram incubadas por 24h em meio RPMI onde o soro fetal bovino (FBS) foi substituído por 5% de um pool PP-PDI ou PR-PDI e a expressão do mRNA de 84 genes foi analisada por *PCR array*. A escala de cores representa o grau de expressão relativa dos genes de interesse em relação ao gene normalizador (β -2-microglobulina) calculada pelo método $\Delta\Delta Ct$ ($n=3$).

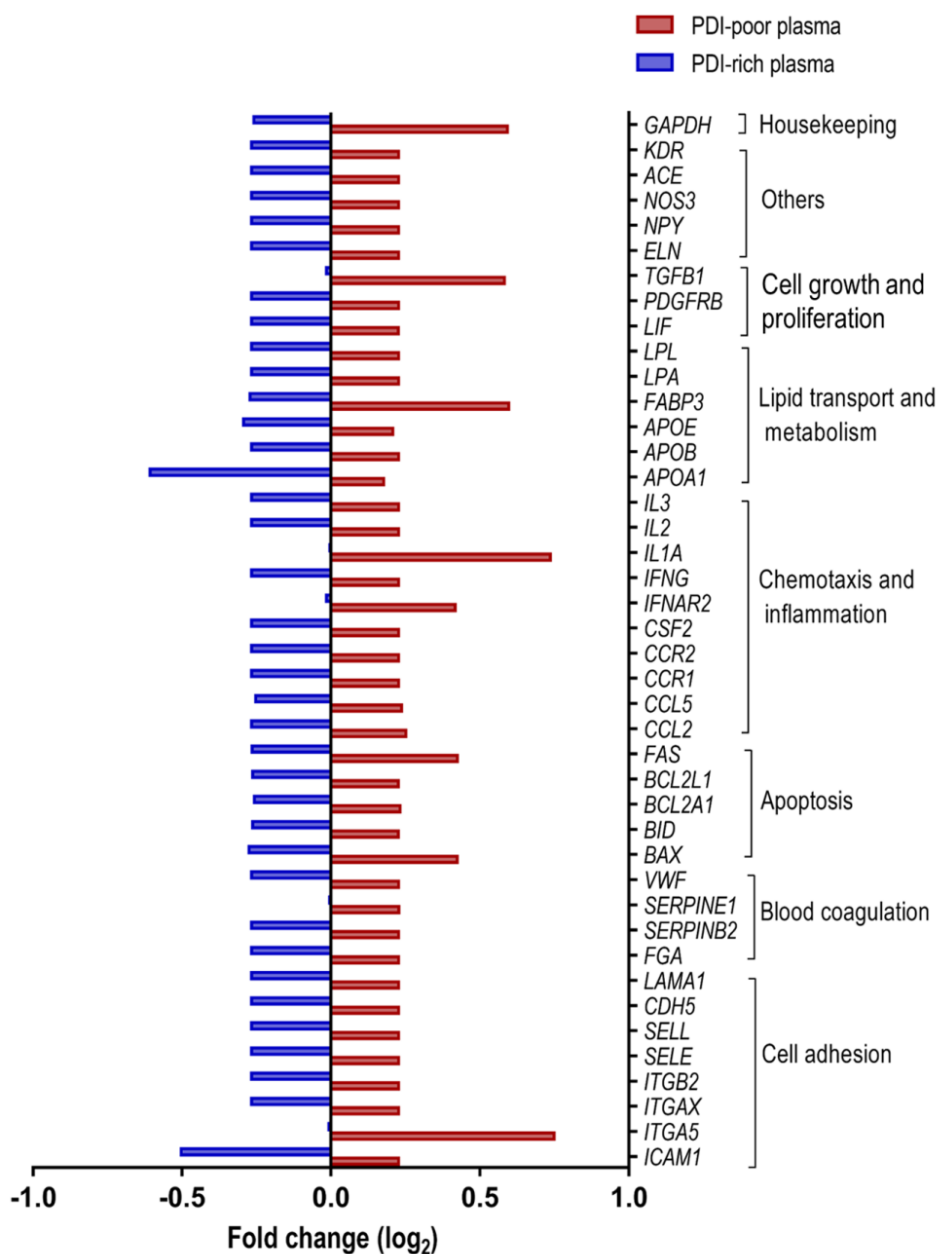


Figura 19 – Expressão do mRNA para os genes que tiveram expressão simultaneamente aumentada nas células expostas aos PP- PDI e diminuída naquelas expostas aos PR-PDI. HUVEC foram incubadas por 24h em meio RPMI no qual o soro fetal bovino (FBS) foi substituído por 5% de um pool de PP-PDI ou PR-PDI e a expressão do mRNA de 84 genes foi analisada por *PCR array*. Os valores para expressão do mRNA estão representados como log₂ do *fold-change* (n= 3).

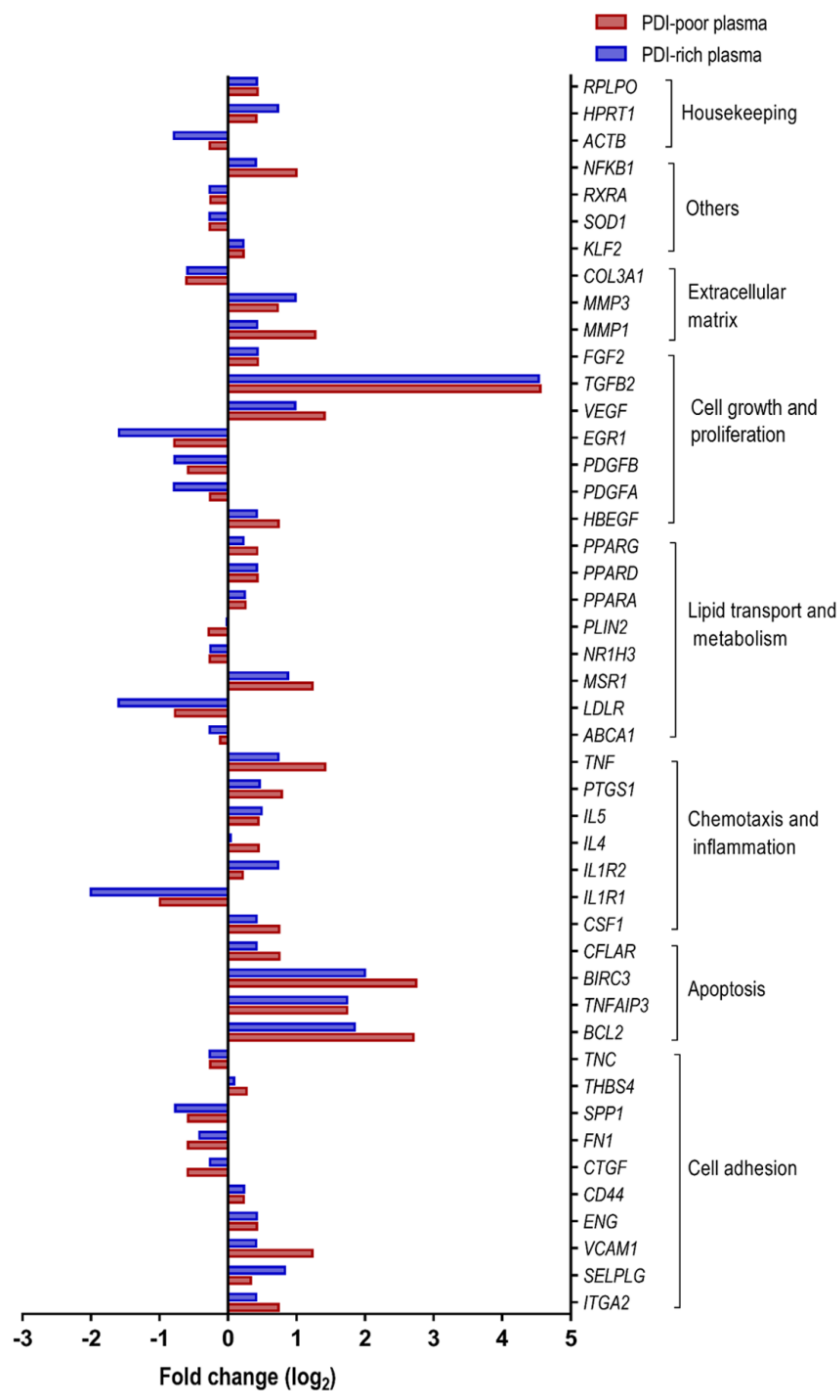


Figura 20 – Expressão do mRNA para os genes que tiveram comportamentos distintos nas células endoteliais expostas aos PP-PDI ou PR-PDI. HUVEC foram incubadas por 24h em meio RPMI no qual o soro fetal bovino (FBS) foi substituído por 5% de um pool de PP-PDI ou PR-PDI e a expressão do mRNA de 84 genes foi analisada por *PCR array*. Os valores para expressão do mRNA estão representados como log₂ do *fold-change* (n= 3).

Para aprofundar essa conexão, analisamos o padrão de secreção de proteínas endoteliais em experimentos executados paralelamente àqueles utilizadas para estudos de expressão gênica. Após 24h de incubação com PP-PDI ou PR-PDI, as culturas de CE foram cuidadosamente lavadas para remoção do meio suplementado com os plasmas ou FBS; em seguida, foram incubadas por um período adicional de 8h com meio livre de FBS ou plasma. Os meios condicionados resultantes foram coletados e submetidos à análise proteômica baseada em espectrometria de massa *shotgun* para geração dos secretomas correspondentes a cada condição.

Foram identificadas 873 proteínas entre todas as condições de incubação (controle-FBS, PP-PDI e PR-PDI). A comparação entre as proteínas presentes nos secretomas após a incubação com os PP-PDI ou PR-PDI, em comparação com Controles-SFB, revelou 26 e 60 proteínas diferencialmente secretadas, respectivamente (Anexos E e F). Consistente com os resultados da Figura 12 no plasma total, o secretoma de CE mostrou expressão proteica diferencial mediante exposição aos PP-PDI vs. PR-PDI (Figura 21), embora neste caso as diferenças tenham sido mais sutis: como as comparações foram realizadas entre cada uma dessas condições vs. controles-FBS, houve menor dissimilaridade entre as vias anotadas. Vias associadas à coagulação sanguínea, por exemplo, apareceram tanto no caso da incubação com PP-PDI ou PR-PDI. Houve o compartilhamento de um certo grau de identidade proteica entre as duas condições, como por exemplo, a presença das cadeias de fibrinogênio e da glicoproteína rica em histidina. Além disso, ambas as condições se associaram com proteínas envolvidas em respostas imuno-inflamatórias e resposta imune antígeno-dependente. No entanto, análises mais detalhadas das proteínas envolvidas nestas e em outras vias e processos, revelaram um perfil diferente de proteínas secretadas após incubação com os PP-PDI vs. PR-PDI, considerando o perfil de ontologia gênica (Figura 22).

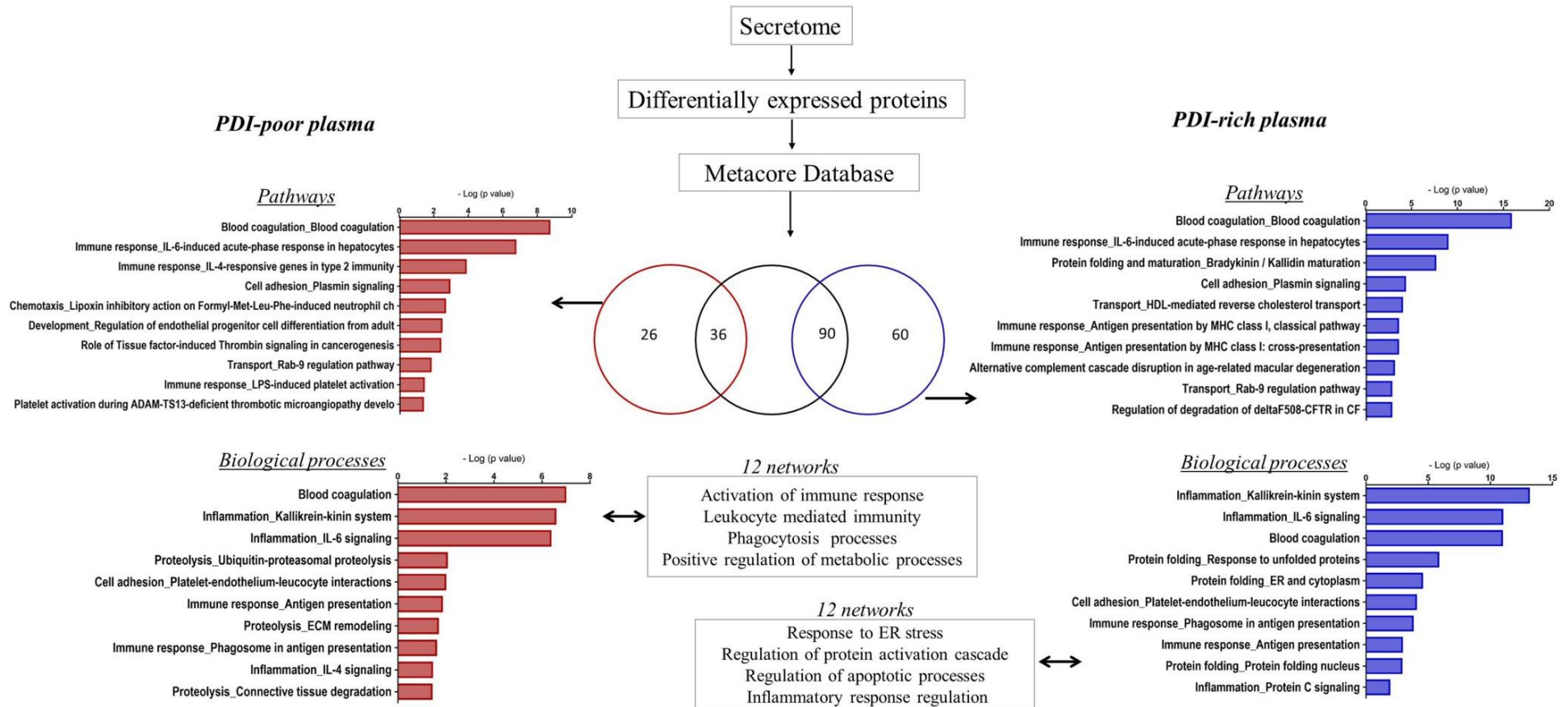
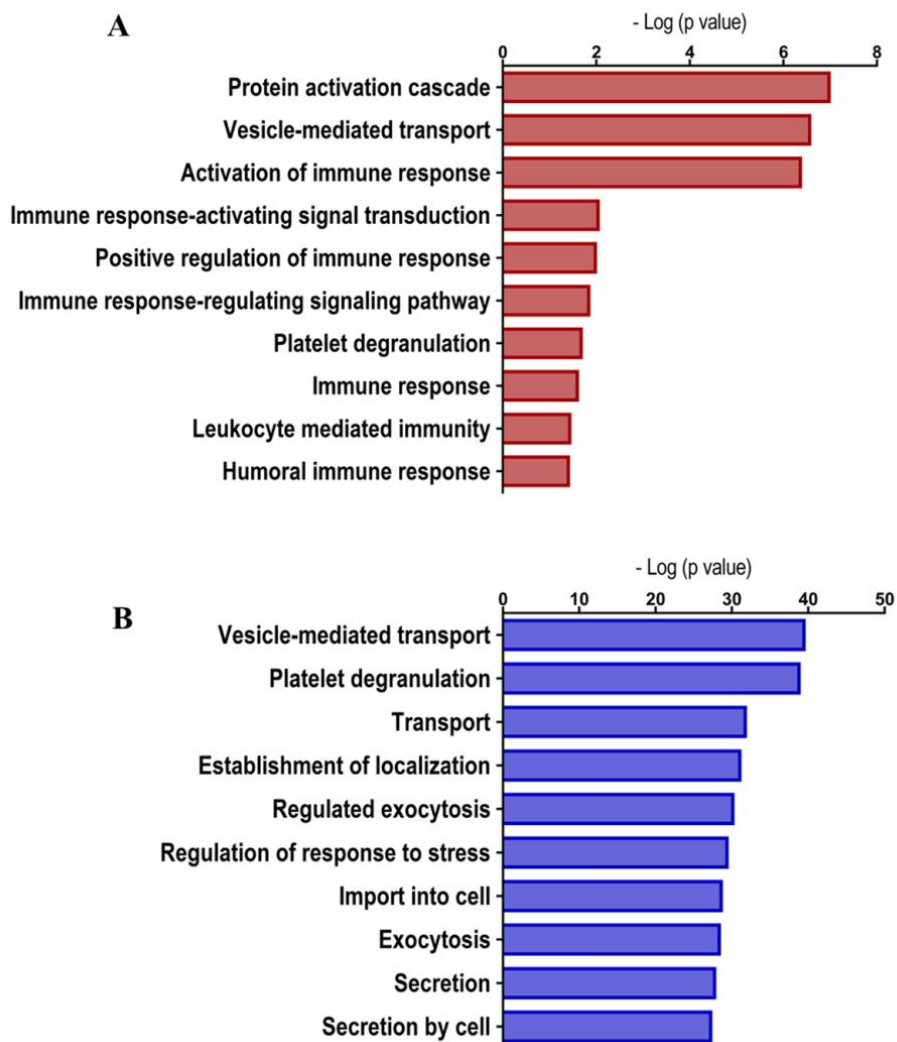


Figura 21 – Proteoma e análise de enriquecimento de vias e processos biológicos de proteínas diferencialmente expressas no secretoma de células endoteliais expostas aos PP-PDI ou PR-PDI. HUVEC foram incubadas por 24h em meio RPMI no qual o soro fetal bovino (FBS) foi substituído por 5% de um pool PP-PDI ou PR-PDI. Os meios condicionados (MC) foram obtidos por incubação com 6 mL de RPMI sem FBS por 8h para análise de secretoma. Os MC foram submetidos à análise de espectrometria de massa (*shotgun*) para a identificação de proteínas diferencialmente expressas nos secretomas de células incubadas com PP-PDI e PR-PDI vs. FBS. Análises adicionais foram realizadas utilizando o *software Metacore* incluindo identificação de vias e processos biológicos associados a cada lista de proteínas ($p < 0,05$).

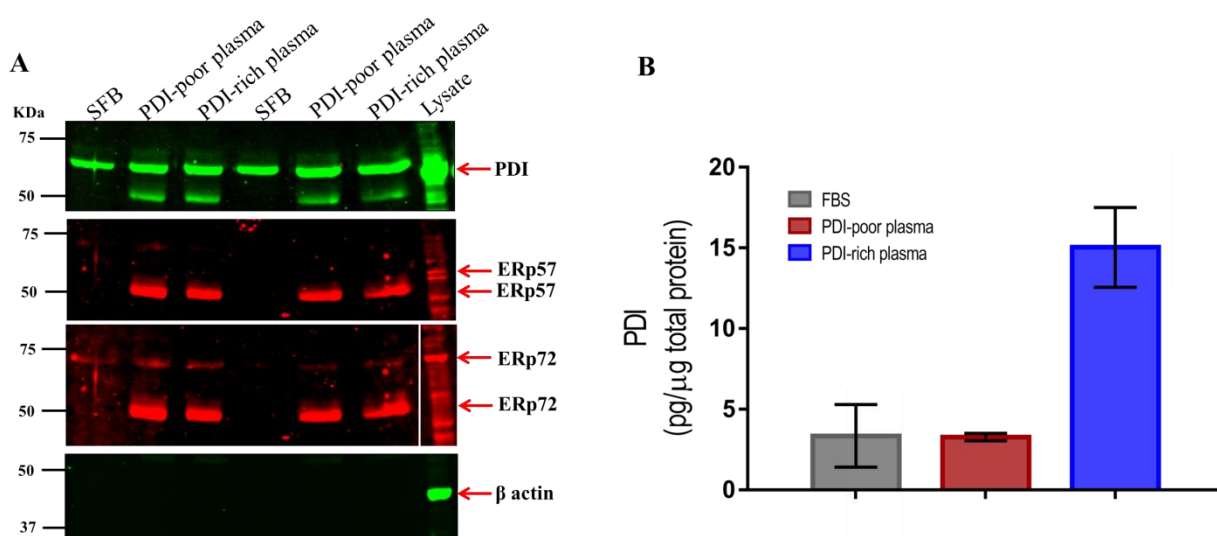


22 – Processos de ontologia genica (*gene ontology, GO*) das proteínas identificadas no secretoma de células endoteliais expostas aos PP-PDI ou PR-PDI. As proteínas de maior expressão nos secretomas de HUVEC foram identificadas por espectrometria de massa e analisadas pelo *software Metacore*. Os principais processos GO ($p < 0,05$) foram representados nos gráficos a seguir. **A.** Secretoma de HUVEC expostas aos PP-PDI ($n = 3$). **B.** Secretoma de HUVEC expostas aos PR-PDI ($n = 3$).

Quando as CE foram expostas aos PR-PDI, a análise do secretoma se associou preferencialmente a processos como enovelamento de proteínas/resposta a proteínas mal enoveladas (p. ex., BiP, chaperona do retículo endoplasmático) e vias de transporte (ex. transtirretina, serotransferrina), enquanto nas CE expostas aos PP-PDI houve associação preferencial com processos de proteólise e regulação de resposta imune (Figura 21 e 22). Nestes experimentos, PDI, ERp57 e ERp72 foram preferencialmente secretadas após

incubação com os PR-PDI, mas não com os PP-PDI em comparação ao controle-SFB. Estes resultados foram confirmados por análise da expressão proteica (Figura 23A) e por ensaio de ELISA, no caso específico da PDI (Figura 23B). É importante ressaltar que não houve evidência de lise celular nestes experimentos, uma vez que a β -actina, um indicador previamente validado como um índice de dano celular no mesmo tipo de célula e ensaio (28), não foi detectada.

Globalmente estes resultados reforçam que o *pool* de PDI detectável no plasma de indivíduos saudáveis se comporta como um marcador de distintos perfis proteômicos associados a relevantes implicações fenotípicas endoteliais; novamente, PP-PDI associaram-se predominantemente a processos de coagulação e resposta imune, enquanto PR-PDI associaram-se a funções de diferenciação e *housekeepings*.



23 – Expressão da PDI e outros membros da família (ERp57, ERp72) no secretoma de células endoteliais. A–B. Amostras de secretoma obtidas a partir de HUVEC incubadas PP-PDI ou PR-PDI e SFB (controle) foram submetidas às seguintes análises: (A) SDS-PAGE e a expressão proteica detectada por anticorpos específicos. A ausência de β -actina nas amostras indica que não houve lise celular durante o preparo dos meios condicionados e as proteínas detectadas são provenientes da secreção celular. Imagens representativas de 3 experimentos independentes (B) detecção da PDI solúvel por ELISA ($n= 3$).

4.7. Correlação entre concentrações de PDI e função de células endoteliais

Após demonstrar perfis proteômicos específicos, bem como fenótipos endoteliais associados aos valores plasmáticos da PDI, questionamos se esses achados poderiam se correlacionar com diferentes respostas funcionais de CE. Para isso, avaliamos o efeito da incubação dos PP-PDI ou PR-PDI na adesão e migração de CE, que são processos cruciais envolvidos na reparação vascular, angiogênese e fisiopatologia de doenças (95). A incubação com PP-PDI inibiu a adesão de CE à fibronectina em cerca de 30% comparado com o controle-FBS ($p < 0,01$) e foi 20% menor em comparação com CE incubadas com PR-PDI ($p < 0,01$). A aparente menor adesão associada aos PR-PDI vs. Controles-SFB não foi estatisticamente significativa (Figura 24). Experimentos análogos utilizando o colágeno do tipo 1 como matriz para adesão não mostraram diferenças entre as CE incubadas com os distintos plasmas (Figura 25). Esses achados estão de acordo com os resultados encontrados na proteômica do plasma total (Figura 12), no qual diversas vias envolvendo as interações adesão celular/célula matriz estavam preferencialmente relacionadas aos PR-PDI.

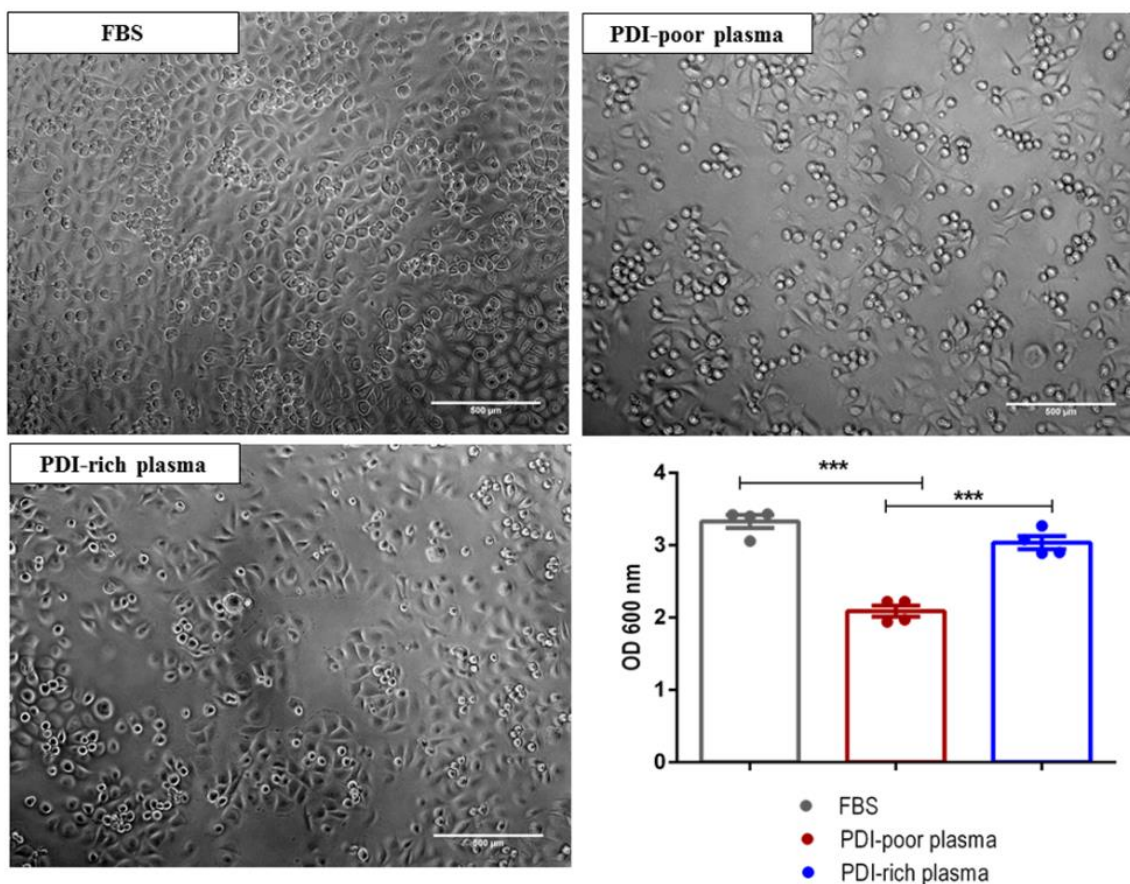


Figura 24 – Efeito da incubação dos PP-PDI e PR-PDI na adesão de células endoteliais à fibronectina. HUVEC (4×10^4 células/mL) foram pré-incubadas com meio de cultura suplementado com 5% dos PP-PDI ou PR-PDI ou SFB por 24h e semeadas em placas cobertas com fibronectina (5 µg/mL) para análise de adesão. Após 1h em estufa, 37°C, as células aderidas foram coradas com cristal violeta seguidas por solubilização em SDS e leitura de densidade óptica em 600 nm ($n= 5$; média \pm erro padrão; (*One-way ANOVA* e pós teste de *Tukey*. *** $P < 0,001$). Fotomicrografias representativas. Barras de escala: 500 µm. Objetiva: 40x.

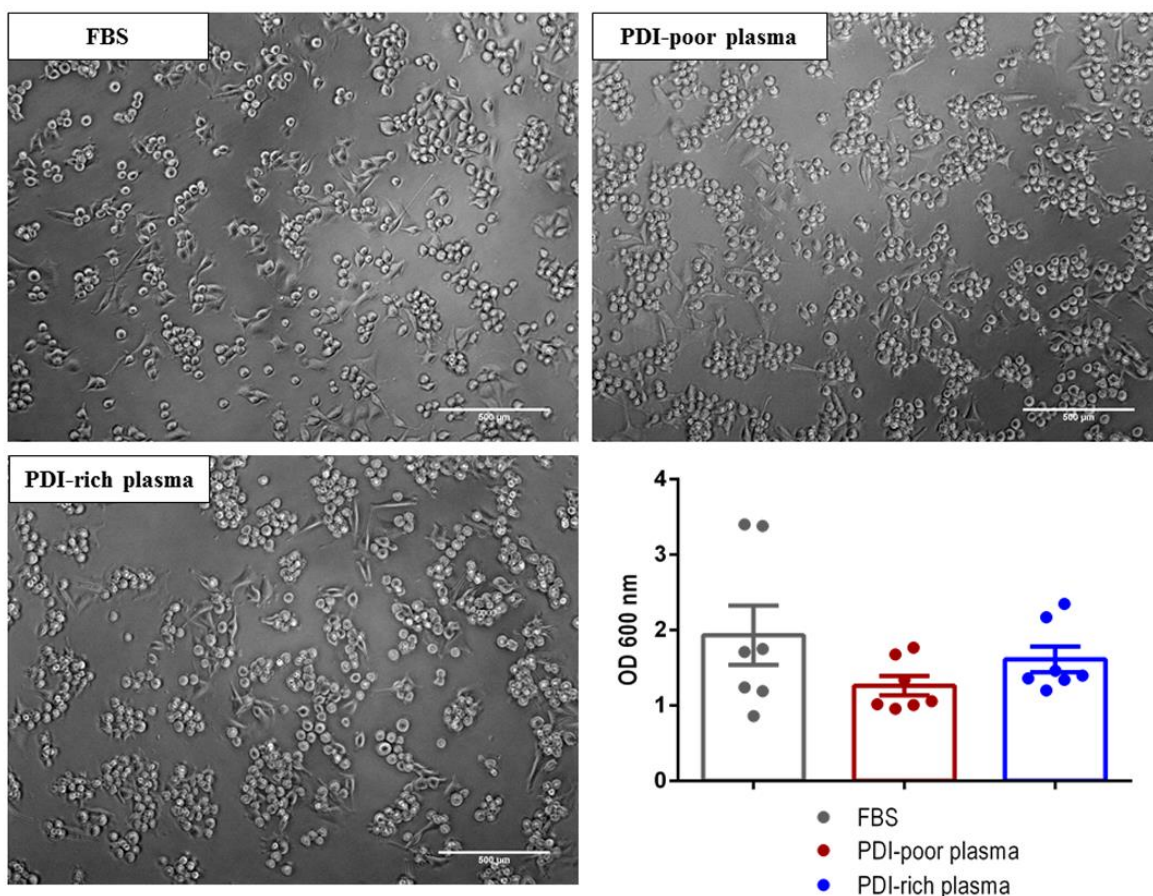


Figura 25 – Efeito da incubação dos PP-PDI e PR-PDI na adesão de células endoteliais ao colágeno do tipo-I. HUVEC (4×10^4 células/mL) foram pré-incubadas com meio de cultura suplementado com 5% dos PP-PDI ou PR-PDI ou SFB por 24h e semeadas em placas cobertas com colágeno do tipo-I (10 µg/mL) para análise de adesão. Após 4h30min, as células aderidas foram coradas com cristal violeta seguidas por solubilização em SDS e leitura de densidade óptica em 600 nm ($n=5$; média \pm erro padrão). Fotomicrografias representativas. Barras de escala: 500 µm. Objetiva: 40x. Sem diferença estatística entre os grupos. *One-way ANOVA* seguido do pós teste de *Tukey*.

Para avaliação adicional das implicações funcionais dos plasmas com distintos valores de PDI, investigamos seus efeitos na migração de CE. Monocamadas de CE foram incubadas como descrito anteriormente (com FBS, PP-PDI ou PR-PDI) durante 24h em ensaios de migração baseado em reparação de lesão (*wound healing*). As CE incubadas com SFB apresentaram maiores taxas de migração e cobriram completamente a área da escarificação após 24h (Figura 26). Por outro lado, o reparo da escarificação foi prejudicado quando as CE foram expostas aos PP-PDI e PR-PDI vs. controles-FBS. O

prejuízo na migração celular foi mais extenso após incubação com os PP-PDI (53% de cobertura na área da ferida) em comparação com as CE incubadas com os PR-PDI (72% de cobertura na área da ferida). Este efeito foi relacionado a uma sequência de eventos intrigantes desencadeados após a incubação com plasmas pobres em PDI. Tanto os PP-PDI quanto os PR-PDI produziram taxas de migração similares até cerca 16h de incubação, correspondentes a 63-65% de cobertura na área total da ferida. No entanto, após esse pico de migração, EC incubadas com PR-PDI continuaram seu processo normal de migração até atingirem um total de 72% da área coberta após 24h, enquanto CE incubadas com PP-PDI continuaram a migrar, mas neste caso, no sentido inverso de migração, expandindo novamente a área da escarificação, de modo que após 24h a área coberta foi de apenas 53% (diminuição em cerca de 12% da cobertura encontrada em 16h de incubação) (Figura 26). A incubação de CE em meio de cultura sem soro (*starved*) mostrou as menores taxas de migração, como esperado. A adição de diferentes concentrações de PDI recombinante a CE submetidas a condição de *starvation* não aumentou suas taxas de migração celular (dados não mostrados); as concentrações de PDI recombinante adicionadas a este ensaio foram nos limites da variação encontrada nos PP-PDI e PR-PDI.

Estes resultados indicam diferentes efeitos associados aos distintos tipos de plasma. Os PR-PDI mantiveram a migração organizada de CE; em contraste, os PP-PDI perturbaram significativamente o padrão de migração.

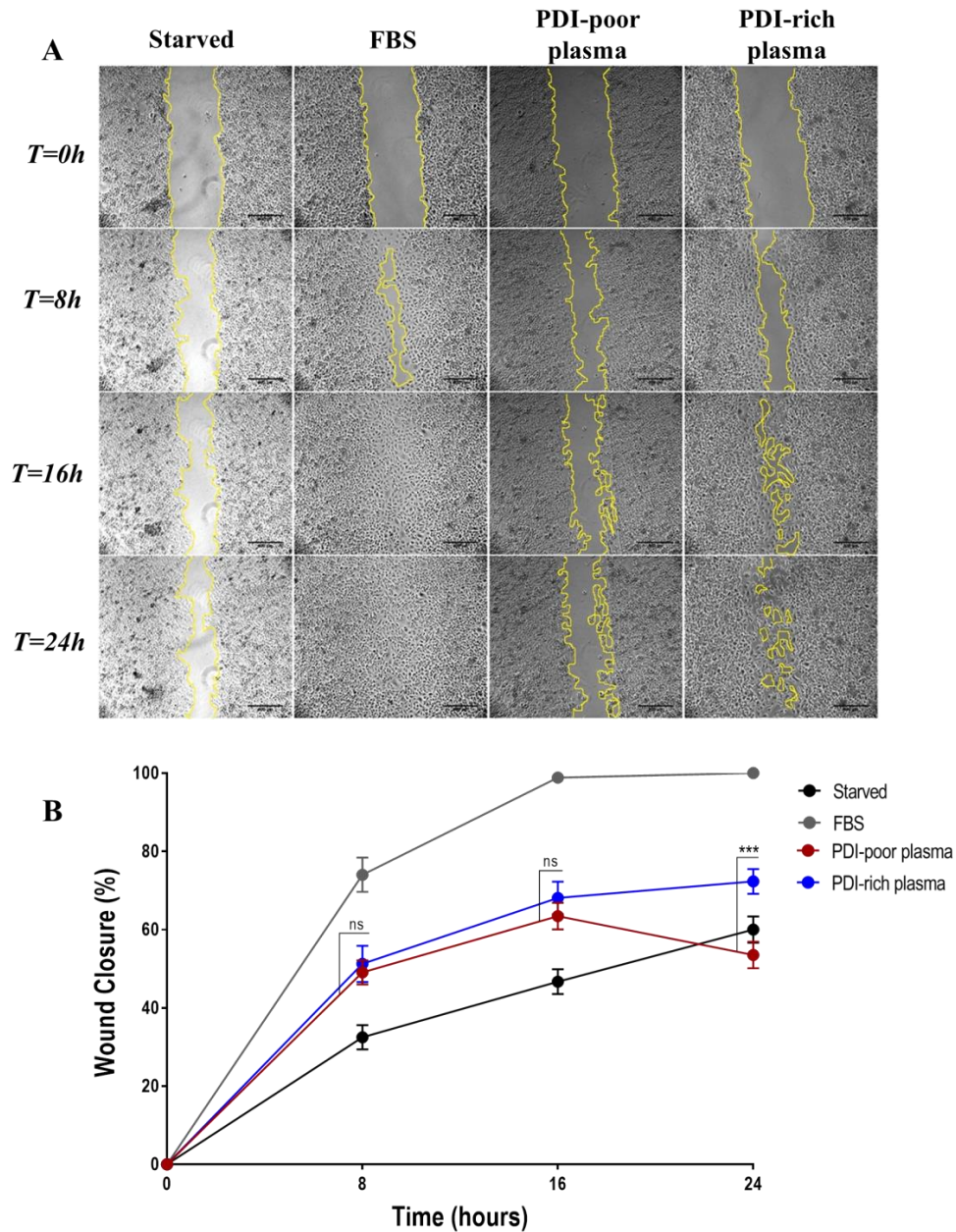


Figura 26 – Efeito da incubação dos PP-PDI e PR-PDI na migração de células endoteliais. HUVEC (2×10^5 células/mL) foram cultivadas por 48h para análise de *wound healing*. Uma escarificação foi criada nas monocamadas celulares com o auxílio de uma ponteira estéril, seguidas de cultivo por 24h adicionais em meio de cultura suplementado como descrito a seguir: Sem FBS (*starved*), com 5% de FBS (controle) e com *pools* de PP-PDI e PR-PDI. **A.** Fotomicrografias representando a migração celular para a área removida em 0h, 8h, 16h e 24h. A zona entre as linhas amarelas indica a área inicial ocupada pela escarificação. Barras de escala: 500 μ m. Objetiva: 5x. **B.** Porcentagem de cobertura da superfície da ferida pela migração de células endoteliais mostradas em (A) ($n = 4$; média \pm erro padrão). *Two-way ANOVA* e pós teste de *Tukey* *** $p < 0.0001$. Para clareza, apenas a estatística entre o grupo de células incubadas com PP-PDI vs. PR-PDI foi mostrada.

Tabela 2. Características clínicas da população de pacientes do biobanco.

<i>n</i>	95
Idade	50,4 ± 1,39
Gênero masculino (%)	40
Colesterol total, mg/dL	279,3 ± 7,5
HDL, mg/mL	48,6 ± 2,3
LDL, mg/dL	204,8 ± 7,3
Triglicerídeos, mg/dL	156,4 ± 9,2
Histórico médico (%)	
Hipertensão	80
Diabetes	28
HF	35
Obesidade	14
Tipo de ECV (%)	
Angina instável	74
IAM	70
AVC	10
Valores de PDI, pg/mL	
Mediana	35
Média	285,1
Percentil 25%	0
Percentil 75%	277

Dados representam média ± erro padrão, exceto quando especificado. HDL: lipoproteína de alta densidade, LDL: lipoproteína de baixa densidade, HF: hipercolesterolemia familiar ECV: evento cardiovascular, IAM: infarto agudo do miocárdio, AVC: acidente vascular cerebral.

5. Discussão

O presente estudo indica que o *pool* detectável da PDI no plasma de indivíduos saudáveis mostra alta variabilidade interindividual, porém baixa variabilidade intraindividual (Figura 8). Importante, os valores de PDI no plasma se comportam como indicadores de perfis proteômicos distintos. Enquanto PR-PDI expressam diferencialmente proteínas relacionadas a diferenciação celular, processamento de proteínas, funções *housekeepings*, entre outras, PP-PDI exibiram diferencialmente um subconjunto de proteínas associadas a coagulação, respostas inflamatórias e imunoativação (Figura 12). Além disso, existe uma estreita correlação entre os valores de PDI no plasma e seus correspondentes perfis proteômicos com o fenótipo e função endotelial. CE incubadas com os dois tipos de plasmas recapitularam perfis de expressão gênica e secreção de proteínas reminiscentes dos observados no proteoma plasmático (Figuras 21 e 22). Além disso, estes perfis proteômicos se traduziram diretamente em respostas funcionais. Os PP-PDI promoveram comprometimento da adesão de CE à fibronectina e perturbaram seu padrão de migração em comparação aos PR-PDI (Figuras 24 e 26).

Em oposição aos nossos resultados, Essex e colaboradores (57) mostraram um *pool* detectável de PDI plasmática, também em doadores saudáveis, medidos por um ensaio de ELISA não comercial (desenvolvido *in-house*). Os valores variaram de 250 a 1000 ng/mL (~4 a 17 nM), um *pool* que ainda é baixo, mas significativamente superior aos valores encontrados em nosso estudo. Seus resultados foram confirmados por análise de expressão proteica após purificação da PDI plasmática em coluna (57). As razões para tais diferenças não são claras, mas provavelmente estão relacionadas às condições do ensaio e às características da população. É importante mencionar que concentrações elevadas de PDI no plasma podem ocorrer em situações em que o *turnover*/morte celular são aumentados, como é o caso de câncer, distúrbios inflamatórios e doenças hematológicas (90), bem como condições ainda desconhecidas. Recentemente, PDI e ERp57 foram reportadas em proteoma plasmático, mas não claramente associadas a risco cardiovascular (98). Nossos dados compõem o primeiro estudo que investigou detalhadamente as concentrações circulantes de proteínas da família da PDI e suas implicações. É provável que além da PDI, outros membros desta família também possam estar presentes em concentrações detectáveis no plasma e se comportarem de maneira semelhante.

Embora nossos resultados indiquem que o *pool* de PDI no plasma está majoritariamente reduzido (Figura 11), a correlação entre estado redox e atividade da PDI merece discussão. A PDI oxidada pode introduz diretamente pontes dissulfeto nos seus substratos e o produto final é a PDI reduzida, que no RE é reoxidada pela Ero1, GSSG, peroxiredoxina-4 ou glutathione peroxidase-7/8 oxidadas (14). A PDI reduzida pode reduzir dissulfetos em substratos, gerando tióis livres (com a PDI oxidada como produto final) ou promover o rearranjo de dissulfetos, com regeneração da PDI reduzida no final (8, 99). Estudos prévios utilizando técnica de captura cinética (*kinetic trapping*) sugerem que diferentes substratos plasmáticos ou plaquetários podem ser modulados por redução ou oxidação mediada pela PDI. Alguns substratos presentes na superfície ou secretados por plaquetas que aparentemente são modulados por redução via PDI incluem fator V da cascata de coagulação (60), anexina V, heparanase, ERp57, calicreína-14, serpina B6, tetranectina, colágeno IV, trombospondina (61); os substratos plasmáticos incluem vitronectina, fator 3 do complemento, proteína de ligação-C4b, alfa-2 macroglobulina, proteína S, proteína rica em histidina, protrombina (59). Os substratos de plaquetas aparentemente modulados por oxidação mediada pela PDI incluem catepsina G, glutarredoxina-1, glicoproteína GP1b e fibrinogênio (61).

Como discutido anteriormente, embora a PDI não se comporte como um sensor redox geral, ela é sensível ao potencial redox do ambiente em que se encontra (efeito local) (8). Com base nesta observação, seria lógico que a PDI no plasma estivesse principalmente oxidada (26) uma vez que o potencial redox dos principais *pools* tiol-dissulfeto plasmáticos, CysSH/CysS-S (-80 ± 9 mV) e GSH/GSSG (-137 ± 9 mV) (4) é mais oxidante que o das cisteínas dos sítios ativos da PDI (-190 ± 10 mV). Esta observação é oposta aos resultados encontrados em nosso estudo. Embora os mecanismos associados a esse aparente paradoxo ainda não estejam claros, essa observação não é surpreendente, pois os principais *pools* tiol-dissulfeto no plasma não estão em equilíbrio, sugerindo que diversos mecanismos podem afetar o equilíbrio entre ambos os *pools* (reduzidos e oxidados) no plasma (4).

Vários mecanismos podem regular a concentração de PDI no plasma e explicar a variabilidade encontrada nas concentrações. Nossos dados (Figura 23 e Anexo F)

mostraram aumento da secreção de PDI por CE expostas aos plasmas ricos em PDI, sugerindo que fatores específicos presentes no plasma podem influenciar a ativação das células e modular a externalização da PDI. Além das CE, a PDI tem papel crítico na trombose, coagulação e função plaquetária (90); plaquetas podem potencialmente secretar PDI após ativação (100, 101) e podem liberar PDI da superfície na ausência de ativação de modo dependente de pH, sem ruptura celular aparente (57). Desta forma, as plaquetas foram sugeridas como possível fonte de PDI no plasma. Em nosso estudo, entretanto, a atividade plaquetária não pode discriminar entre indivíduos com PP-PDI ou PR-PDI. Outra observação relevante é que, de maneira geral, houve diminuição na PDI solúvel após a ativação plaquetária. Atribuímos este achado ao sequestro da PDI por seus potenciais substratos plaquetários e plasmáticos descritos previamente (15, 59, 61). De fato, a PDI é retida na superfície de plaquetas pela ligação à integrina β_3 (26, 50), e pode se associar a vários substratos do plasma. Um efeito semelhante pode provavelmente ocorrer com outros substratos circulantes da PDI que se ligam à superfície de CE; esses efeitos podem ser responsáveis em parte pela variabilidade encontrada nas concentrações de PDI. Estudos com metodologia semelhante sugeriram que PDI detectada em plasma não é significativamente alterada pela ativação plaquetária em indivíduos saudáveis, porém a secreção da PDI por plaquetas ativadas de indivíduos hemofílicos é aumentada (102). A concentração geral da *pecPDI* é muito inferior ao total de PDI intracelular, porém pode ser aumentada após superexpressão forçada. De fato, aortas de camundongos que superexpressam a PDI, modelo desenvolvido em nosso laboratório, secretam concentrações elevadas de PDI na ausência de lesão (38). Além disso, é importante ressaltar que o presente estudo avaliou indivíduos saudáveis e possivelmente processos associados a doenças podem causar oscilação nos valores de PDI no plasma. Em casos de lesão vascular e inflamação em curso, por exemplo, pode-se antecipar um aumento da PDI circulante devido a morte e extravasamento celular (90). Finalmente, observações recentes sugerem que polimorfismos de nucleotídeos únicos são uma fonte substancial de variabilidade em proteínas plasmáticas (103), um fato que merece investigação mais profunda no caso da PDI.

Um resultado importante foi o efeito funcional contrastante dos PR-PDI e PP-PDI na migração de CE. Os primeiros sustentaram uma migração organizada enquanto os últimos desorganizaram significativamente o padrão de migração (Figura 26). Em ambos os casos, estes efeitos estão provavelmente associados aos perfis proteicos que compõem cada tipo de plasma e não diretamente aos valores de PDI em si, considerando que os valores médios da PDI no plasma têm concentrações pM/nM, que contrastam com concentrações muito mais elevadas dos seus substratos plasmáticos, p.ex.: glicoproteína rica em histidina (HRG) (100–150 µg/mL) (82), outras tiol proteínas (400– 600 µM) e tióis de baixo peso molecular (7,3–9,0 µM para glutationa; 202–281 µM para cisteína) (4). Assim, propomos que a PDI se comporte como um indicador/marcador do tipo e/ou quantidade dos substratos a ela ligados. Considerando a complexidade e o espectro dinâmico de proteínas plasmáticas, vários potenciais candidatos podem mediar esses efeitos. Um exemplo é a HRG, presente no plasma humano em altas concentrações e diferencialmente expressa em ambos os tipos de plasma, bem como no secretoma de CE (5,6 e 3,5 vezes, respectivamente). Estudos reportam o envolvimento da HRG na perda de aderência de macrófagos (104) e células T (105), além de efeitos estimuladores e/ou inibitórios na angiogênese (82). Adicionalmente, os plasmas pobres em PDI exibiram proteínas associadas à reparação e migração tecidual, como revelado pela análise de ontologia gênica (Figura 13).

Recentemente, um estudo proteômico com indivíduos que tiveram aneurisma de aorta abdominal mostrou que a expressão de diversas proteínas plasmáticas no trombo luminal e na parede do aneurisma se correlacionou a maiores taxas de crescimento do aneurisma. Ao contrário, proteínas associadas a matriz extracelular, síntese de proteínas, resposta antioxidante e citoesqueleto foram negativamente associadas às taxas de crescimento (106). Curiosamente, várias proteínas identificadas nesse estudo se sobrepõem aos nossos resultados proteômicos em secretoma de CE (27 proteínas) e plasma total (7 proteínas). Estes incluem proteínas relacionadas à resposta imune e complemento, bem como coagulação (p. ex.: cininogênio e plasminogênio), todas correlacionadas positivamente com o crescimento do aneurisma e em nosso estudo mais expressas em amostras de PP-PDI. Por outro lado, as proteínas do RE associadas ao envelhecimento de

proteínas, como PDI e ERp72, que se associaram negativamente ao crescimento do aneurisma (106) estavam positivamente reguladas no secretoma de CE expostas aos PR-PDI. Esse paralelo entre os dois estudos revela que os valores de PDI no plasma podem funcionar como um discriminador de perfis plasmáticos com potencial de se associar a doenças vasculares.

Neste sentido, a PDI soma-se a outras tiol proteínas pelo potencial para constituir um marcador de doenças. Estas incluem a glutathiona peroxidase-3, quiescina sulfidril oxidase, tioredoxina/tiorredoxina redutase, glutaredoxina-1 (4), S-nitrosoglutationa redutase (107). Entretanto, com exceção da associação descrita entre glutaredoxina-1 e permeabilidade endotelial (108), os efeitos endoteliais funcionais relacionados a tais proteínas permanecem desconhecidos, assim como sua correlação com perfis plasmáticos específicos. Até o presente, nosso estudo é o primeiro a descrever diferentes perfis proteômicos no plasma e fenótipos endoteliais correspondentes relacionados a (dis)função vascular. Desta forma, nossos resultados sugerem novas formas de entender como biomarcadores, no geral, e especificamente biomarcadores redox, podem interagir com processos envolvidos em mecanismos de doenças.

É importante mencionar que uma clara limitação dos nossos resultados está diretamente associada ao tipo de população e o pequeno número de indivíduos, o que não permite nenhuma inferência sobre a capacidade de os valores de PDI atuarem como um biomarcador. No entanto, para obter potenciais indícios sobre esse questionamento, investigamos a concentração de PDI em amostras de plasma ($n=95$) coletadas em um biobanco previamente descrito e validado. A população incluía indivíduos com doença vascular clinicamente evidente (71, 72) cujas características clínicas estão descritas na Tabela 2. Pacientes que apresentaram eventos cardiovasculares documentados, como infarto agudo do miocárdio, angina instável e acidente vascular cerebral mostraram valores médios de PDI circulante significativamente mais baixos em relação a indivíduos saudáveis (Figura 27). Este resultado sugere que menores valores de PDI circulante podem ter correlação geral com risco cardiovascular aumentado em pacientes com aterosclerose. Mais uma vez a concentração plasmática de PDI não se correlacionou com a maioria dos fatores de risco tradicionais (gênero, LDL, HDL, triglicérides, diabetes, índice de massa corpórea),

com exceção da idade ($p < 0,05$) (Anexo G). Esses resultados estão de acordo com nossos estudos proteômicos e funcionais em indivíduos saudáveis, porém reforçamos que estudos adicionais são necessários para avaliar a aplicabilidade geral da PDI plasmática como biomarcador de risco.

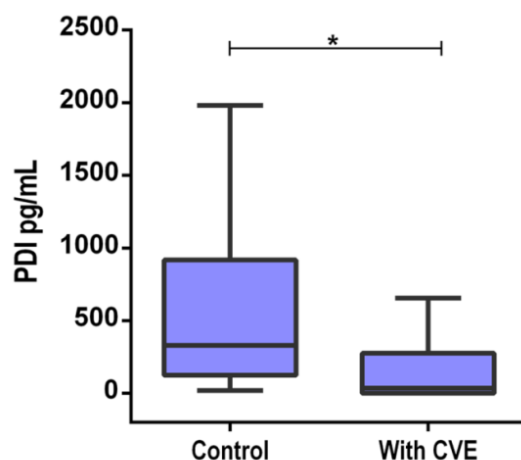


Figura 27 – Concentração circulante da PDI em pacientes do biobanco e controles saudáveis. Concentração da PDI em amostras de plasma armazenadas no biobanco do programa Hipercol Brasil que apresentaram eventos cardiovasculares (angina instável, infarto agudo do miocárdio e acidente cerebrovascular) ($n=95$) e controles ($n=35$) foi analisada por ELISA. Boxplots representam medianas e intervalos interquartis. $p < 0,05$ de acordo com o teste Mann-Whitney.

Globalmente nossos dados reafirmam que proteínas plasmáticas, incluindo PDI, podem se comportar como contribuidores, bem como marcadores acessíveis de eventos/condições associadas a doenças (109), fornecendo evidências de que esses conceitos também se estendem a indivíduos saudáveis. O avanço na identificação de marcadores/indicadores adequados, particularmente aqueles relacionados à PDI, pode fornecer informações sobre mecanismos fisiopatológicos envolvendo perfis de proteínas plasmáticas associados à vasculatura/endotélio. O *pool* de PDI identificado neste estudo pode dar suporte a um inovador conceito de biópsia líquida endotelial.

6. Conclusões

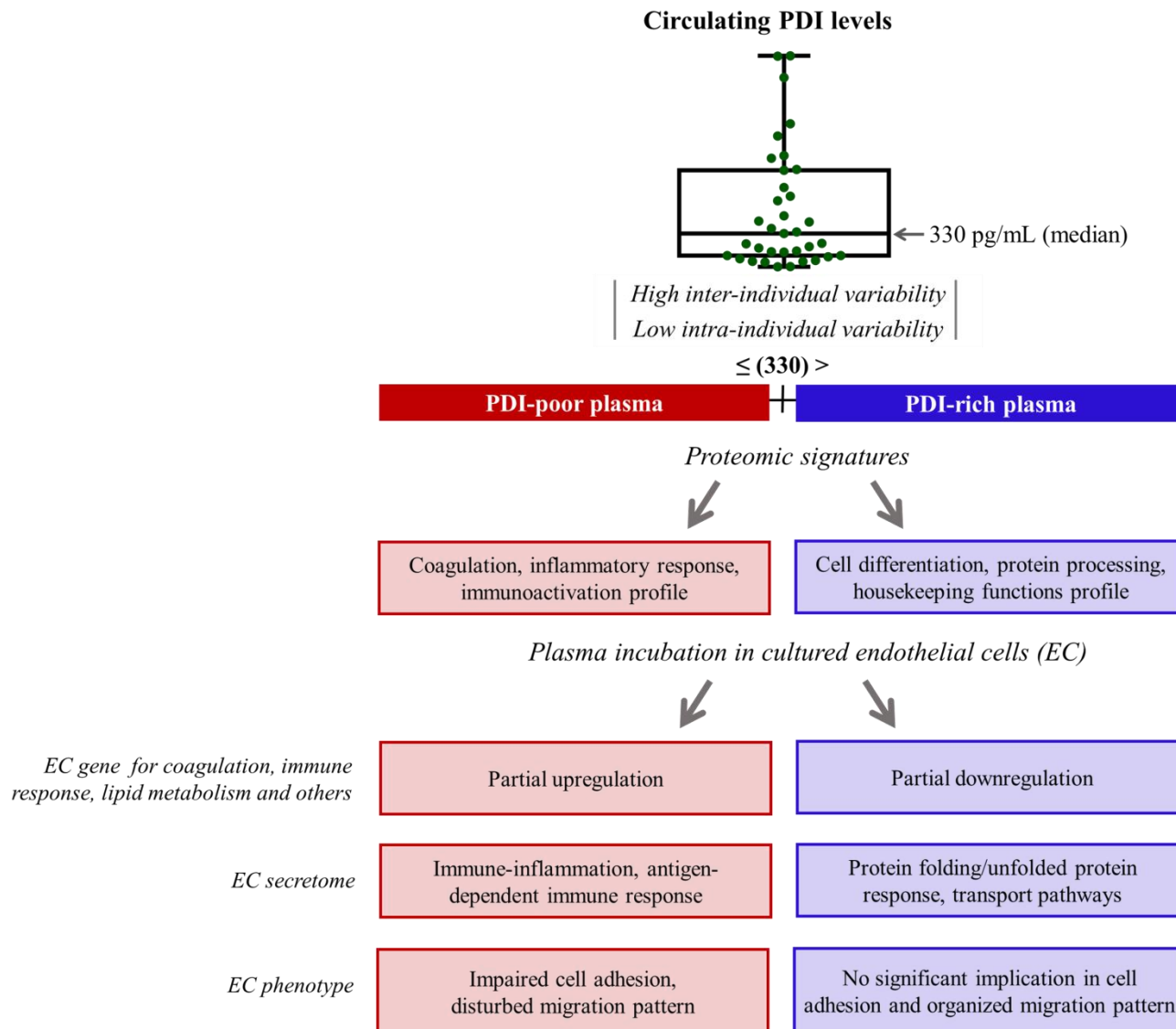


Figura 28 – Resumo gráfico.

Os principais resultados do presente estudo podem ser resumidos como:

- Validamos um ensaio para detecção dos valores plasmáticos da PDI em indivíduos saudáveis, os quais mostraram alta variabilidade interindividual, porém baixa variabilidade intraindividual.
- PR-PDI (> mediana) associaram-se a um perfil proteômico relacionado à diferenciação celular, processamento de proteínas, funções *housekeepings*, enquanto

PP-PDI foram diferencialmente associados à coagulação, respostas inflamatórias e imuno-ativação.

- Células endoteliais incubadas com PP-PDI ou PR-PDI recapitularam padrões de expressão gênica e secreção de proteínas semelhantes aos perfis correspondentes no plasma.
- PP-PDI prejudicaram adesão à fibronectina e migração de células endoteliais em comparação aos PR-PDI.
- Pacientes que tiveram eventos cardiovasculares apresentaram valores mais baixos de PDI no plasma vs. indivíduos saudáveis.

A análise destes resultados permite concluir que proteínas redox presentes no plasma, como proteínas da família da PDI, são candidatas a indicadores/marcadores de assinaturas proteicas associadas ao fenótipo e função de células endoteliais, de um modo reminescente de uma biópsia líquida endotelial. Este é o primeiro estudo descrevendo a PDI como um indicador de assinaturas proteômicas plasmáticas específicas, promovendo distintos fenótipos endoteliais, com potenciais implicações em medicina personalizada.

7. Anexos

Anexo A

Termo de consentimento livre e esclarecido assinado pelos voluntários da pesquisa

1

**HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE
DE SÃO PAULO-HCFMUSP**

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

I. Dados da pesquisa:

Título da pesquisa: Dissulfeto Isomerase Proteica como via integrativa entre estresse oxidativo e resposta de proteínas mal-enoveladas na reparação vascular

Título do subprojeto: Dissulfeto Isomerase Proteica como um marcador independente de risco para trombose, disfunção endotelial e/ou inflamação em doença aterosclerótica em modelos experimentais e em pacientes com Hipercolesterolemia familiar e controles

Protocolo de pesquisa: 1086/09; SDC: 3334/09/085

Pesquisador principal: Prof. Francisco Rafael Martins Laurindo

Pesquisador responsável: Prof. Dr. Francisco Rafael Martins Laurindo

Pesquisador executante: Percillia Victória Santos de Oliveira

Departamento/Instituto: Laboratório de Biologia Vascular - Instituto do Coração – Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo - HCFMUSP

De acordo com a resolução [466/2012](#) os seguintes conteúdos devem fazer parte das explicações sobre a pesquisa.

II. Descrição e objetivo da pesquisa:

Você está sendo convidado a participar de um estudo para investigar a Dissulfeto isomerase Proteica (PDI) presente no plasma como um marcador de risco para eventos relacionados a doenças cardiovasculares em pacientes com hipercolesterolemia familiar e controles. Será efetuada uma coleta de sangue que será realizada no laboratório de análises clínicas do Incor, com enfermeiros treinados, para a realização das seguintes análises: colesterol total e frações, triglicérides, hemograma completo e valores plasmáticos da PDI e outros marcadores conhecidos de inflamação e tendência trombogênica. Quando o resultado ficar pronto entraremos em contato com o(a) senhor(a) para entregarmos os resultados.

III. Procedimentos que serão realizados e métodos que serão empregados:

A Coleta de sangue será realizada por profissionais enfermeiros treinados do Laboratório de análises clínicas (Instituto do Coração).

- É necessário estar em jejum de 12 horas.

Rubrica sujeito da pesquisa ou responsável:

Rubrica responsável pelo projeto:

TCLE 3ª versão de 05/05/2016

- Será efetuada coleta de 20 mL de sangue em 4 tubos de 5ml cada. A coleta seguirá todas as normas de higiene e segurança estabelecidas.

IV. Riscos esperados:

Riscos: Risco mínimo.

V. Esclarecimentos dados pelo pesquisador sobre garantias do sujeito da pesquisa consignado

- 1 Estas informações estão sendo fornecidas para sua participação voluntária neste estudo, que tem como objetivo investigar a PDI presente no plasma como um marcador de risco para eventos relacionados a doenças cardiovasculares;
- 2 Você pode, a qualquer momento, fazer a retirada do consentimento, ou seja, você pode desistir do estudo em qualquer momento sem que haja qualquer prejuízo.
- 3 Direito de confidencialidade: para manter sua privacidade, será fornecido um número de registro à sua amostra. Dessa forma, seu nome será mantido no mais absoluto sigilo e não será divulgado em nenhum momento da pesquisa.
- 4 Os pacientes que concordarem em participar do projeto deverão assinar o termo de consentimento livre e esclarecido.
- 5 Você assinará as duas vias do termo de consentimento livre esclarecido e receberá por direito uma das vias para controle pessoal.
- 6 Em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis pela pesquisa para esclarecimento de eventuais dúvidas. O principal investigador é o **Prof. Francisco Rafael Martins Laurindo** que poderá ser encontrado no endereço Av. Dr. Enéas Carvalho de Aguiar, nº 44, 9º andar . Telefone: 11 2661-5185. Se o Sr.(a) tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa (CEP) – Rua Ovídio Pires de Campos, 225 – 5º andar – tel:(11)2661-7585/ (11)2661 1548 (11)2661 1549 FAX: 2661-6442– E-mail: cappesq.adm@hc.fm.usp.br
- 7 É garantida a liberdade da retirada de consentimento a qualquer momento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na Instituição;
- 8 Direito de confidencialidade – As informações obtidas serão analisadas em conjunto com outros pacientes, não sendo divulgada a identificação de nenhum paciente;
- 9 Direito de ser mantido atualizado sobre os resultados parciais das pesquisas, quando em estudos abertos, ou de resultados que sejam do conhecimento dos pesquisadores;

Rubrica sujeito da pesquisa ou responsável:

Rubrica responsável pelo projeto:

TCLE 3ª versão de 05/05/2016

- 10 Despesas e compensações: não há despesas pessoais para o participante em qualquer fase do estudo, incluindo exames e consultas. Também não há compensação financeira relacionada à sua participação. Se existir qualquer despesa adicional, ela será absorvida pelo orçamento da pesquisa.
- 11 O material coletado será processado e armazenado em banco/repositório do InCor. O mesmo será utilizado para as análises dos marcadores. Após os testes, o plasma será mantido no banco para futuros estudos envolvendo este ou outro projeto relacionado à Hipercolesterolemia Familiar e a PDI. O material será mantido no banco/repositório durante os três anos de vigor do Projeto, mas este prazo poderá ser maior caso o Projeto seja renovado.

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO-HCFMUSP

Eu discuti com o Prof. Francisco Rafael Martins Laurindo ou a Percillia Victória Santos de Oliveira sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido, ou no meu atendimento neste Serviço.

Assinatura do paciente/representante legal

Data ____/____/____

Assinatura da testemunha

Data ____/____/____

Para casos de pacientes menores de 18 anos, analfabetos, semi-analfabetos ou portadores de deficiência auditiva ou visual.

(Somente para o responsável do projeto)

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste paciente ou representante legal para a participação neste estudo.

Assinatura do responsável pelo estudo

Data ____/____/____

Rubrica sujeito da pesquisa ou responsável:

Rubrica responsável pelo projeto:

TCLE 3ª versão de 05/05/2016

DADOS DE IDENTIFICAÇÃO DO SUJEITO DA PESQUISA OU RESPONSÁVEL LEGAL

1. NOME:.....

DOCUMENTO DE IDENTIDADE Nº:..... SEXO: M () F () DATA NASCIMENTO:/...../.....

ENDEREÇO Nº APTO:

BAIRRO: CIDADE:.....

CEP:..... TELEFONE: DDD (.....)

2. RESPONSÁVEL LEGAL

NATUREZA (grau de parentesco, tutor, curador etc.)

DOCUMENTO DE IDENTIDADE:.....SEXO: M () F () DATA NASCIMENTO:/...../.....

ENDEREÇO: Nº APTO:

BAIRRO: CIDADE:

CEP: TELEFONE: DDD (.....)

Rubrica sujeito da pesquisa ou responsável:

Rubrica responsável pelo projeto:

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DADOS SOBRE A PESQUISA

1. TÍTULO DO PROTOCOLO DE PESQUISA: Dissulfeto Isomerase proteica como via integrativa entre estresse oxidativo e resposta de proteínas mal-enoveladas na reparação vascular

TÍTULO DO SUBPROJETO: Dissulfeto Isomerase Proteica como um marcador independente de risco para trombose, disfunção endotelial e/ou inflamação em doença aterosclerótica em modelos experimentais e em pacientes com Hipercolesterolemia familiar e controles

PROTOCOLO DE PESQUISA: 1086/09; SDC: 3334/09/085

2. PESQUISADOR: Prof. Francisco Rafael Martins Laurindo

NÚMERO INSCRIÇÃO CRM: 61539

CARGO/FUNÇÃO: Diretor do Laboratório de Biologia Vascular

Pesquisador responsável: Francisco Rafael Martins Laurindo

NÚMERO INSCRIÇÃO CRM: 35341

CARGO/FUNÇÃO: Pesquisador responsável

Pesquisador executante: Percília Victória Santos de Oliveira

CARGO/FUNÇÃO: pesquisador executante

UNIDADE DO HCFMUSP: Instituto do Coração – Laboratório de Biologia Vascular

3. AVALIAÇÃO DO RISCO DA PESQUISA:

RISCO MÍNIMO (X)

RISCO MÉDIO ()

RISCO BAIXO ()

RISCO MAIOR ()

4. DURAÇÃO DA PESQUISA: 3 anos

Rubrica sujeito da pesquisa ou responsável:

Rubrica responsável pelo projeto:

TCLE 3ª versão de 05/05/2016

Anexo B

Proteínas diferencialmente expressas no plasma total de indivíduos com PP-PDI.

<i>Entry</i>	<i>Gene name</i>	<i>Number of peptides</i>	<i>Max Fold change</i>	<i>Log₂ Fold change</i>	<i>Description</i>
P00747	PLG	102	1.13	0.18	Plasminogen
P01042	KNG1	81	1.07	0.10	Kininogen-1
P04196	HRG	59	1.48	0.57	Histidine-rich glycoprotein
P02748	C9	55	1.34	0.42	Complement component C9
P04003	C4BPA	48	1.55	0.63	C4b-binding protein alpha chain
P07357	C8A	41	1.30	0.38	Complement component C8 alpha chain
Q06033	ITIH3	39	1.41	0.50	Inter-alpha-trypsin inhibitor heavy chain H3
P10909	CLU	39	1.13	0.18	Clusterin
P20742	PZP	66	2.23	1.16	Pregnancy zone protein
P00748	F12	32	1.48	0.57	Coagulation factor XII
P02750	LRG1	34	1.35	0.43	Leucine-rich alpha-2-glycoprotein
P03951	F11	33	1.09	0.12	Coagulation factor XI
Q9NYC9	DNAH9	31	1.20	0.26	Dynein heavy chain 9, axonemal
P07360	C8G	20	1.39	0.48	Complement component C8 gamma chain
P21359	NF1	32	1.31	0.39	Neurofibromin
H0Y390	MACF1	19	1.13	0.18	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5 (Fragment)
Q9ULT8	HECTD1	19	1.11	0.15	E3 ubiquitin-protein

					ligase HECTD1
P02647	APOA1	18	1.38	0.46	Apolipoprotein A-I
Q13315	ATM	21	1.10	0.14	Serine-protein kinase ATM
P08571	CD14	13	1.20	0.26	Monocyte differentiation antigen CD14
Q9NZP8	C1RL	20	1.13	0.18	Complement C1r subcomponent-like protein
Q9UP83	COG5	11	1.20	0.26	Conserved oligomeric Golgi complex subunit 5
P38398	BRCA1	17	1.16	0.21	Breast cancer type 1 susceptibility protein
G3V3A5	TTC6	13	1.19	0.25	Tetratricopeptide repeat protein 6
O14646	CHD1	9	1.17	0.23	Chromodomain- helicase-DNA- binding protein 1
P21817	RYR1	9	1.07	0.10	Ryanodine receptor 1
Q9P212	PLCE1	7	1.17	0.23	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1
Q6ZN30	BNC2	12	1.18	0.24	Zinc finger protein basonuclin-2
Q15124	PGM5	11	1.21	0.28	Phosphoglucomutase- like protein 5
P07358	C8B	48	1.48	0.57	Complement component C8 beta chain
P63261	ACTA1	17	1.23	0.30	Actin, alpha skeletal muscle
P63261	ACTG1	22	1.26	0.33	Actin, cytoplasmic 2
Q7Z443	PKD1L3	9	1.13	0.18	Polycystic kidney disease protein 1-like 3
Q7Z333	SETX	7	1.19	0.25	Probable helicase senataxin

Q9BYW2	SETD2	6	1.10	0.14	Histone-lysine N-methyltransferase SETD2
Q9P0V3	SH3BP4	6	1,09	0.12	SH3 domain-binding protein 4
Q96SK3	ZNF607	5	1.31	0.39	Zinc finger protein 607
P28827	PTPRM	5	1.27	0.34	Receptor-type tyrosine-protein phosphatase mu
O94913	PCF11	5	1.16	0.21	Pre-mRNA cleavage complex 2 protein Pcf11
Q8N531	FBXL6	5	1.10	0.14	F-box/LRR-repeat protein 6
P20794	MAK	4	1.31	0.39	Serine/threonine-protein kinase MAK
Q8IYU8	MICU2	4	1.25	0.32	Calcium uptake protein 2, mitochondrial
Q13822	ENPP2	4	1.20	0.26	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2
Q9UPP1	PHF8	4	1.19	0.25	Histone lysine demethylase PHF8
Q96P48	ARAP1	4	1.15	0.20	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1
Q3BBV0	NBPF1	3	1.46	0.55	Neuroblastoma breakpoint family member 1
Q9Y6D9	MAD1L1	3	1.11	0.15	Mitotic spindle assembly checkpoint protein MAD1
Q9H7T0	CATSPERB	5	1.14	0.19	Cation channel sperm-associated protein subunit beta

Q14563	SEMA3A	4	1.22	0.29	Semaphorin-3A
Q96IL0		4	1.09	0.12	Uncharacterized protein
P14618	PKM	3	1.97	0.98	Pyruvate kinase PKM
Q13474	DRP2	3	1.73	0.79	Dystrophin-related protein 2
Q96Q07	BTBD9	3	1.68	0.75	BTB/POZ domain-containing protein 9
G3V3G9		3	1.34	0.42	Uncharacterized protein
P45983	MAPK8	3	1.31	0.39	Mitogen-activated protein kinase 8
Q7Z4N2	TRPM1	3	1.27	0.34	Transient receptor potential cation channel subfamily M member 1
Q9UDV7	ZNF282	3	1.19	0.25	Zinc finger protein 282
Q15418	RPS6KA1	3	1.19	0.25	Ribosomal protein S6 kinase alpha-1
Q9UF33	EPHA6	2	1.76	0.82	Ephrin type-A receptor 6
A0A0K0K1C4	TRBV27	2	1.76	0.82	Protein TRBV27 (Fragment)
H0YI58	ERGIC2	2	1.72	0.78	Endoplasmic reticulum-Golgi intermediate compartment protein 2 (Fragment)
Q99497	PARK7	2	1.21	0.28	Protein deglycase DJ-1
Q99574	SERPINI1	2	1.16	0.21	Neuroserpin
O00423	EML1	2	1.13	0.18	Echinoderm microtubule-associated protein-like 1
H7C0L5	ITIH4	80	1.15	0.20	Inter-alpha-trypsin inhibitor heavy chain H4 (Fragment)

Anexo C

Proteínas diferencialmente expressas no plasma total de indivíduos com PR-PDI.

<i>Entry</i>	<i>Gene name</i>	<i>Number of peptides</i>	<i>Max Fold change</i>	<i>Log₂ Fold change</i>	<i>Description</i>
P51884	LUM	24	1.29	0.36	Lumican
Q13439	GOLGA4	24	1.19	0.25	Golgin subfamily A member 4
P02753	RBP4	16	1.16	0.21	Retinol-binding protein 4
Q9Y6R7	FCGBP	9	1.26	0.33	IgGFC-binding protein
P33151	CDH5	9	1.17	0.23	Cadherin-5
O94887	FARP2	18	1.35	0.43	FERM, RhoGEF and pleckstrin domain-containing protein 2
Q02224	CENPE	13	1.11	0.15	Kinesin-like protein
O94964	SOGA1	11	2.42	1.28	Protein SOGA1
O75376	NCOR1	11	1.20	0.26	Nuclear receptor corepressor 1
Q15811	ITSN1	10	1.18	0.24	Intersectin-1
P21580	TNFAIP3	9	1.12	0.16	Tumor necrosis factor alpha-induced protein 3
P02745	C1QA	8	1.39	0.48	Complement C1q subcomponent subunit A
Q99973	TEP1	8	1.12	0.16	Telomerase protein component 1
Q96JG6	VPS50	7	1.21	0.28	Syndetin
Q9ULC5	ACSL5	8	1.26	0.33	Long chain fatty acid CoA ligase 5
Q3V6T2	CCDC88A	8	1.26	0.33	Girdin
P13591	NCAM1	6	1.38	0.46	Neural cell adhesion molecule 1
A2CJ06	DYTN	10	1.23	0.30	Dystrotelin
O60716	CTNND1	6	1.35	0.43	Catenin delta-1
P42702	LIFR	6	1.35	0.42	Leukemia inhibitory factor receptor
P32004	L1CAM	5	1.26	0.32	Neural cell adhesion molecule L1
Q9NX02	NLRP2	5	1.24	0.31	NACHT, LRR and PYD domains-containing protein 2
Q5H9F3	BCORL1	4	1.14	0.19	BCL-6 corepressor-like protein 1

Q8N1I0	DOCK4	8	1.18	0.24	Dedicator of cytokinesis protein 4 (Fragment)
Q9Y4D7	PLXND1	6	1.94	0.96	Plexin-D1
O14523	C2CD2L	6	1.49	0.58	C2 domain-containing protein 2-like
Q96JN8	NEURL4	6	1.20	0.26	Neuralized-like protein 4
Q86XX4	FRAS1	5	1.31	0.39	Extracellular matrix protein FRAS1
Q9UJF2	RASAL2	5	1.29	0.37	Ras GTPase-activating protein nGAP
B2RXF5	ZBTB42	4	2.96	1.57	Zinc finger and BTB domain-containing protein 42
Q9UHW9	SLC12A6	4	1.27	0.34	Solute carrier family 12 member 6
Q92614	MYO18A	4	1.22	0.29	Unconventional myosin-XVIIIa
P14920	DAO	4	1.19	0.25	D-amino-acid oxidase
Q96IT1	ZNF496	3	1.57	0.64	Zinc finger protein 496
Q02045	MYL5	3	1.27	0.34	Myosin light chain 5
Q9BQE5	APOL2	3	1.23	0.29	Apolipoprotein L2
P78410	BTN3A2	3	1.16	0.20	Butyrophilin subfamily 3 member A2 (Fragment)
P78524	ST5	3	1.10	0.14	Suppression of tumorigenicity 5 protein
Q68DK2	ZFYVE26	6	1.26	0.33	Zinc finger FYVE domain-containing protein 26
Q9NTJ3	SMC4	4	1.74	0.80	Structural maintenance of chromosomes protein
Q9BWW1	BOC	4	1.34	0.42	Brother of CDO
P05023	ATP1A1	4	1.29	0.37	Sodium/potassium-transporting ATPase subunit alpha-1
P40121	CAPG	3	1.44	0.53	Macrophage-capping protein
Q8NE71	ABCF1	3	1.31	0.39	ATP-binding cassette sub-family F member 1
P61769	B2M	3	1.28	0.34	Beta-2-microglobulin
P16070	CD44	3	1.27	0.34	CD44 antigen

Q8N972	ZNF709	3	1.24	0.31	Zinc finger protein 709
P13535	MYH8	3	1.17	0.21	Myosin-8
Q68CZ6	HAUS3	3	1.15	0.20	HAUS augmin-like complex subunit 3
Q16587	ZNF74	2	2.39	1.25	Zinc finger protein 74
P13349	MYF5	2	1.73	0.78	Myogenic factor 5
H3BNF6	MVP	2	1.67	0.74	Major vault protein (Fragment)
Q6UXF7	CLEC18B	2	1.54	0.62	C-type lectin domain family 18 member B
Q9HBD1	RC3H2	2	1.37	0.45	Roquin-2
Q13257	MAD2L1	2	1.32	0.40	Mitotic spindle assembly checkpoint protein MAD2A
Q9BUU2	METTL22	2	1.28	0.34	Methyltransferase-like protein 22
O75356	ENTPD5	2	1.25	0.31	Ectonucleoside triphosphate diphosphohydrolase 5
Q8N183	NDUFAF2	2	1.24	0.31	Mimitin, mitochondrial
P08151	GLI1	2	1.23	0.30	Zinc finger protein GLI1
P27482	CALML3	2	1.21	0.26	Calmodulin-like protein 3

Anexo D

Lista de genes amplificados por PCR (*RT² profiler PCR array*).

Classification	Symbol	GeneBank	Description
Cell adhesion			
	ITGA2	NM_002203	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
	ITGA5	NM_002205	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
	ITGAX	NM_000887	Integrin, alpha X (complement component 3 receptor 4 subunit)
	ITGB2	NM_000211	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
	LAMA1	NM_005559	Laminin, alpha 1
	CTGF	NM_001901	Connective tissue growth factor
	CDH5	NM_001795	Cadherin 5, type 2 (vascular endothelium)
	CD44	NM_000610	CD44 molecule (Indian blood group)
	FN1	NM_002026	Fibronectin 1
	SELE	NM_000450	Selectin E
	SELL	NM_000655	Selectin L
	SELPLG	NM_003006	Selectin P ligand
	SPP1	NM_000582	Secreted phosphoprotein 1
	ICAM1	NM_000201	Intercellular adhesion molecule 1
	VCAM1	NM_001078	Vascular cell adhesion molecule 1
	THBS4	NM_003248	Thrombospondin 4
	TNC	NM_002160	Tenascin C
	ENG	NM_000118	Endoglin
Blood coagulation			
	FGA	NM_000508	Fibrinogen alpha chain
	SERPINB2	NM_002575	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
	SERPINE1	NM_000602	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
	VWF	NM_000552	Von Willebrand factor
Chemotaxis and inflammation			
	CCL2	NM_002982	Chemokine (C-C motif) ligand 2
	CCL5	NM_002985	Chemokine (C-C motif) ligand 5
	CCR1	NM_001295	Chemokine (C-C motif) receptor 1
	CCR2	NM_001123396	Chemokine (C-C motif) receptor 2

CSF1	NM_000757	Colony stimulating factor 1 (macrophage)
CSF2	NM_000758	Colony stimulating factor 2 (granulocyte-macrophage)
IFNAR2	NM_000874	Interferon (alpha, beta and omega) receptor 2
IFNG	NM_000619	Interferon, gamma
IL1A	NM_000575	Interleukin 1, alpha
IL1R1	NM_000877	Interleukin 1 receptor, type I
IL1R2	NM_004633	Interleukin 1 receptor, type II
IL2	NM_000586	Interleukin 2
IL3	NM_000588	Interleukin 3 (colony-stimulating factor, multiple)
IL4	NM_000589	Interleukin 4
IL5	NM_000879	Interleukin 5 (colony-stimulating factor, eosinophil)
PTGS1	NM_000962	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
TNF	NM_000594	Tumor necrosis factor
Apoptosis		
BAX	NM_004324	BCL2-associated X protein
BCL2	NM_000633	B-cell CLL/lymphoma 2
BCL2A1	NM_004049	BCL2-related protein A1
BCL2L1	NM_138578	BCL2-like 1
BID	NM_001196	BH3 interacting domain death agonist
BIRC3	NM_001165	Baculoviral IAP repeat containing 3
CFLAR	NM_003879	CASP8 and FADD-like apoptosis regulator
FAS	NM_000043	Fas (TNF receptor superfamily, member 6)
TNFAIP3	NM_006290	Tumor necrosis factor, alpha-induced protein 3
Lipid transport and metabolism		
ABCA1	NM_005502	ATP-binding cassette, sub-family A (ABC1), member 1
APOA1	NM_000039	Apolipoprotein A-I
APOB	NM_000384	Apolipoprotein B (including Ag(x) antigen)
APOE	NM_000041	Apolipoprotein E
FABP3	NM_004102	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
LDLR	NM_000527	Low density lipoprotein receptor
LPA	NM_005577	Lipoprotein, Lp(a)
LPL	NM_000237	Lipoprotein lipase
MSR1	NM_002445	Macrophage scavenger receptor 1
NR1H3	NM_005693	Nuclear receptor subfamily 1, group H, member 3
PLIN2	NM_001122	Perilipin 2
PPARA	NM_005036	Peroxisome proliferator-activated receptor alpha

	PPARD	NM_006238	Peroxisome proliferator-activated receptor delta
	PPARG	NM_015869	Peroxisome proliferator-activated receptor gamma
Cell growth and proliferation			
	HBEGF	NM_001945	Heparin-binding EGF-like growth factor
	LIF	NM_002309	Leukemia inhibitory factor (cholinergic differentiation factor)
	PDGFA	NM_002607	Platelet-derived growth factor alpha polypeptide
	PDGFB	NM_002608	Platelet-derived growth factor beta polypeptide
	PDGFRB	NM_002609	Platelet-derived growth factor receptor, beta polypeptide
	TGFB1	NM_000660	Transforming growth factor, beta 1
	TGFB2	NM_003238	Transforming growth factor, beta 2
	FGF2	NM_002006	Fibroblast growth factor 2 (basic)
	EGR1	NM_001964	Early growth response 1
	VEGFA	NM_003376	Vascular endothelial growth factor A
Extracellular matrix			
	COL3A1	NM_000090	Collagen, type III, alpha 1
	MMP1	NM_002421	Matrix metalloproteinase 1 (interstitial collagenase)
	MMP3	NM_002422	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)
Others			
	NOS3	NM_000603	Nitric oxide synthase 3 (endothelial cell)
	KDR	NM_002253	Kinase insert domain receptor (a type III receptor tyrosine kinase)
	ELN	NM_000501	Elastin
	KLF2	NM_016270	Kruppel-like factor 2 (lung)
	SOD1	NM_000454	Superoxide dismutase 1, soluble
	NPY	NM_000905	Neuropeptide Y
	RXRRA	NM_002957	Retinoid X receptor, alpha
	NFKB1	NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
	ACE	NM_000789	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
Housekeepings			
	ACTB	NM_001101	Actin, beta
	B2M	NM_004048	Beta-2-microglobulin
	GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase
	HPRT1	NM_000194	Hypoxanthine phosphoribosyltransferase 1
	RPLP0	NM_001002	Ribosomal protein, large, P0

Anexo E

**Proteínas diferencialmente expressas no secretoma de CE incubadas com PP-
PDI vs. controles (SFB).**

<i>Entry</i>	<i>Gene name</i>	<i>Number of peptides</i>	<i>Max Fold change</i>	<i>Log₂ Fold change</i>	<i>Description</i>
P01857	IGHG1	28	33.0	5.0	Immunoglobulin heavy constant gamma 1
P31150	GDI1	7	3.9	1.9	Rab GDP dissociation inhibitor alpha
P01009	SERPINA1	23	60.2	6.1	Alpha-1-antitrypsin
P02749	APOH	6	3.0	1.6	Beta-2-glycoprotein 1
P20618	PSMB1	5	29.7	4.9	Proteasome subunit beta type-1
P02763	ORM1	5	17.9	4.2	Alpha-1-acid glycoprotein 1
P28070	PSMB4	2	Infinity	-	Proteasome subunit beta type-4
Q14624	ITIH4	3	34.5	5.1	Inter-alpha-trypsin inhibitor heavy chain H4
P01861	IGHG4	8	17	4.1	Immunoglobulin heavy constant gamma 4
P01859	IGHG2	12	11.1	3.5	Immunoglobulin heavy constant gamma 2
Q99988	GDF15	7	38.0	5.2	Growth/differentiation factor 15
P02679	FGG	15	72.1	6.2	Fibrinogen gamma chain
P01834	IGKC	10	47.0	5.6	Immunoglobulin kappa constant
P01860	IGHG3	11	13.1	3.7	Immunoglobulin heavy constant gamma 3
P01877	IGHA2	7	15.0	3.9	Immunoglobulin heavy constant alpha 2
P05155	SERPING1	10	3.7	1.9	Plasma protease C1 inhibitor
P19652	ORM2	5	14.3	3.8	Alpha-1-acid glycoprotein 2
P07339	CTSD	3	18.0	4.2	Cathepsin D
P02671	FGA	8	24.6	4.6	Fibrinogen alpha chain
P04196	HRG	19	49.9	5.6	Histidine-rich glycoprotein
P02774	GC	11	6.6	2.7	Vitamin D-binding protein
P02675	FGB	18	7.1	2.8	Fibrinogen beta chain
P61160	ACTR2	3	11.7	3.5	Actin-related protein 2
P01876	IGHA1	13	7.6	2.9	Immunoglobulin heavy constant alpha 1
P30086	7 PEBP1	7	17.8	4.2	Phosphatidylethanolamine-binding protein 1
P28066	PSMA5	2	32.4	5.0	Proteasome subunit alpha type-5

Anexo F

Proteínas diferencialmente expressas no secretoma de CE incubadas com PR-PDI vs. controles (SFB).

<i>Entry</i>	<i>Gene name</i>	<i>Number of peptides</i>	<i>Max Fold change</i>	<i>Log₂ Fold change</i>	<i>Description</i>
P00738	HP	23	25.2	4.7	Haptoglobin
P02679	FGG	16	344.1	8.4	Fibrinogen gamma chain
P00739	HPR	12	62.4	6.0	Haptoglobin-related protein
P01009	SERPINA1	25	35.6	5.2	Alpha-1-antitrypsin
P02671	FGA	8	53.4	5.7	Fibrinogen alpha chain
P31150	GDI1	7	4.0	2.0	Rab GDP dissociation inhibitor alpha
P19338	NCL	8	7.0	2.8	Nucleolin
P02647	APOA1	13	11.2	3.5	Apolipoprotein A-I
P01871	IGHM	17	28.1	4.8	Immunoglobulin heavy constant mu
P25311	AZGP1	5	3.8	1.9	Zinc-alpha-2-glycoprotein
P01861	IGHG4	9	44	5.5	Immunoglobulin heavy constant gamma 4
P04217	A1BG	7	7.9	3.0	Alpha-1B-glycoprotein
P01023	A2M	35	8.1	3.0	Alpha-2-macroglobulin
P01860	IGHG3	12	44.8	5.5	Immunoglobulin heavy constant gamma 3
P02787	TF	57	18.4	4.2	Serotransferrin
P02766	TTR	6	10.5	3.4	Transthyretin
Q92598	HSPH1	6	7.7	3.0	Heat shock protein 105 kDa
P06312	IGKV4	2	5.7	2.5	Immunoglobulin kappa variable 4-1
P01764	IGHV3	3	15.3	3.9	Immunoglobulin heavy variable 3-23
P00747	PLG	16	17.7	4.1	Plasminogen
P68871	HBB	6	15.2	3.9	Hemoglobin subunit beta
P01859	IGHG2	13	10.9	3.4	Immunoglobulin heavy constant gamma 2
P02763	ORM1	6	37.1	5.2	Alpha-1-acid glycoprotein 1
Q96PD2	DCBLD2	3	4.1	2.0	Discoidin_ CUB and LCCL domain-containing protein 2
P39687	ANP32A	2	91.7	6.5	Acidic leucine-rich nuclear phosphoprotein 32 family member A
Q06830	PRDX1	8	10.3	3.4	Peroxiredoxin-1

P11021	HSPA5	18	1.3	0.3	Endoplasmic reticulum chaperone BiP
P04196	HRG	19	11.3	3.5	Histidine-rich glycoprotein
P09651	HNRNPA1	3	5.8	2.5	Heterogeneous nuclear ribonucleoprotein A1
P69905	HBA1	4	6.3	2.6	Hemoglobin subunit alpha
P01042	KNG1	5	13.8	3.8	Kininogen-1
P02790	HPX	18	2.7	1.4	Hemopexin
P00505	GOT2	11	2.5	1.3	Aspartate aminotransferase_mitochondrial
P01780	IGHV3-7	3	5.9	2.6	Immunoglobulin heavy variable 3-7
P04083	ANXA1	4	4.3	2.1	Annexin A1
P27797	CALR	9	3.7	1.9	Calreticulin
P02675	FGB	18	13.9	3.8	Fibrinogen beta chain
P02749	APOH	7	3.8	1.9	Beta-2-glycoprotein 1
P50395	GDI2	9	2.2	1.1	Rab GDP dissociation inhibitor beta
P01008	SERPINC1	12	2.2	1.1	Antithrombin-III
Q9NQC3	RTN4	2	88.1	6.5	Reticulon-4
P22392	NME2	8	2.0	1.0	Nucleoside diphosphate kinase B
P14625	HSP90B1	5	4.1	2.0	Endoplasmin
P23528	CFL1	5	1.7	0.8	Cofilin-1
P05155	SERPING1	10	2.9	1.6	Plasma protease C1 inhibitor
P00450	CP	5	7.8	3.0	Ceruloplasmin
P01876	IGHA1	15	6.4	2.7	Immunoglobulin heavy constant alpha 1
P07237	P4HB	19	1.6	0.7	Protein disulfide-isomerase
P01857	IGHG1	26	112.0	6.8	Immunoglobulin heavy constant gamma 1
P01619	IGKV3D-20	2	5.8	2.5	Immunoglobulin kappa variable 3D-20
P13667	PDIA4	10	1.2	0.2	Protein disulfide-isomerase A4
P30101	PDIA3	11	1.5	0.6	Protein disulfide-isomerase A3
P01834	IGKC	10	46.4	5.5	Immunoglobulin kappa constant
P0DOX7	N/A	9	46.4	5.5	Immunoglobulin kappa light chain
P09493	TPM1	5	2.9	1.5	Tropomyosin alpha-1 chain
P26641	EEF1G	5	3.8	1.9	Elongation factor 1-gamma
P22626	HNRNPA2B1	4	3.1	1.7	Heterogeneous nuclear ribonucleoproteins A2/B1
P00751	CFB	5	12.8	3.7	Complement factor B
P02774	GC	12	1.8	0.8	Vitamin D-binding protein
P11021	HSPA5	18	1.2	0.3	78 kDa glucose-regulated protein

Anexo G

Associação valores plasmáticos da PDI e fatores de risco tradicionais em pacientes que tiveram eventos cardiovasculares (biobanco).

Variáveis independentes	β	P valor
Idade	-3.01	0.003*
Gênero	-0.30	0.76
IMC, Kg/m ²	0.53	0.6
Colesterol total, mg/dL	1.23	0.22
HDL, mg/dL	-0.78	0.43
LDL, mg/dL	1.05	0.30
Triglicérides, mg/dL	-0.25	0.80
Diabetes	-0.6	0.52

IMC: índice de massa corpórea, HDL: lipoproteína de alta densidade, LDL: lipoproteína de baixa densidade. β : coeficiente de regressão, *p<0.05

8. Referências

1. Go YM, Jones DP. Thiol/disulfide redox states in signaling and sensing. *Crit Rev Biochem Mol Biol*. 2013;48(2):173-81.
2. Jones DP, Sies H. The Redox Code. *Antioxid Redox Signal*. 2015;23(9):734-46.
3. Jones DP. Redefining oxidative stress. *Antioxid Redox Signal*. 2006;8(9-10):1865-79.
4. Oliveira PVS, Laurindo FRM. Implications of plasma thiol redox in disease. *Clin Sci (Lond)*. 2018;132(12):1257-80.
5. Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol*. 2008;4(5):278-86.
6. Stöcker S, Van Laer K, Mijuskovic A, Dick TP. The Conundrum of Hydrogen Peroxide Signaling and the Emerging Role of Peroxiredoxins as Redox Relay Hubs. *Antioxid Redox Signal*. 2018;28(7):558-73.
7. Stöcker S, Maurer M, Ruppert T, Dick TP. A role for 2-Cys peroxiredoxins in facilitating cytosolic protein thiol oxidation. *Nat Chem Biol*. 2018;14(2):148-55.
8. Soares Moretti AI, Martins Laurindo FR. Protein disulfide isomerases: Redox connections in and out of the endoplasmic reticulum. *Arch Biochem Biophys*. 2017;617:106-19.
9. Appenzeller-Herzog C, Ellgaard L. The human PDI family: versatility packed into a single fold. *Biochim Biophys Acta*. 2008;1783(4):535-48.
10. Hatahet F, Ruddock LW. Protein disulfide isomerase: a critical evaluation of its function in disulfide bond formation. *Antioxid Redox Signal*. 2009;11(11):2807-50.
11. Galligan JJ, Petersen DR. The human protein disulfide isomerase gene family. *Hum Genomics*. 2012;6:6.
12. Ellgaard L, Ruddock LW. The human protein disulphide isomerase family: substrate interactions and functional properties. *EMBO Rep*. 2005;6(1):28-32.
13. Parakh S, Atkin JD. Novel roles for protein disulphide isomerase in disease states: a double edged sword? *Front Cell Dev Biol*. 2015;3:30.
14. Laurindo FR, Pescatore LA, Fernandes DeC. Protein disulfide isomerase in redox cell signaling and homeostasis. *Free Radic Biol Med*. 2012;52(9):1954-69.
15. Flaumenhaft R. Advances in vascular thiol isomerase function. *Curr Opin Hematol*. 2017;24(5):439-45.

16. Bekendam RH, Bendapudi PK, Lin L, Nag PP, Pu J, Kennedy DR, et al. A substrate-driven allosteric switch that enhances PDI catalytic activity. *Nat Commun.* 2016;7:12579.
17. Wang L, Wang X, Wang CC. Protein disulfide-isomerase, a folding catalyst and a redox-regulated chaperone. *Free Radic Biol Med.* 2015;83:305-13.
18. Zapun A, Creighton TE, Rowling PJ, Freedman RB. Folding in vitro of bovine pancreatic trypsin inhibitor in the presence of proteins of the endoplasmic reticulum. *Proteins.* 1992;14(1):10-5.
19. Ohba H, Harano T, Omura T. Intracellular and intramembranous localization of a protein disulfide isomerase in rat liver. *J Biochem.* 1981;89(3):889-900.
20. Klappa P, Hawkins HC, Freedman RB. Interactions between protein disulphide isomerase and peptides. *Eur J Biochem.* 1997;248(1):37-42.
21. Irvine AG, Wallis AK, Sanghera N, Rowe ML, Ruddock LW, Howard MJ, et al. Protein disulfide-isomerase interacts with a substrate protein at all stages along its folding pathway. *PLoS One.* 2014;9(1):e82511.
22. Hetz C, Mollereau B. Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. *Nat Rev Neurosci.* 2014;15(4):233-49.
23. Molinari M, Galli C, Piccaluga V, Pieren M, Paganetti P. Sequential assistance of molecular chaperones and transient formation of covalent complexes during protein degradation from the ER. *J Cell Biol.* 2002;158(2):247-57.
24. Lee SO, Cho K, Cho S, Kim I, Oh C, Ahn K. Protein disulphide isomerase is required for signal peptide peptidase-mediated protein degradation. *EMBO J.* 2010;29(2):363-75.
25. Meirelles T, Araujo TL, Nolasco P, Moretti AI, Guido MC, Debbas V, et al. Fibrillin-1 mg Δ (l μ n) Marfan syndrome mutation associates with preserved proteostasis and bypass of a protein disulfide isomerase-dependent quality checkpoint. *Int J Biochem Cell Biol.* 2016;71:81-91.
26. Flaumenhaft R, Furie B. Vascular thiol isomerases. *Blood.* 2016;128(7):893-901.
27. Römer RA, Wells SA, Emilio Jimenez-Roldan J, Bhattacharyya M, Vishweshwara S, Freedman RB. The flexibility and dynamics of protein disulfide isomerase. *Proteins.* 2016;84(12):1776-85.

28. Araujo TLS, Zeidler JD, Oliveira PVS, Dias MH, Armelin HA, Laurindo FRM. Protein disulfide isomerase externalization in endothelial cells follows classical and unconventional routes. *Free Radic Biol Med.* 2017;103:199-208.
29. Essex DW. Redox control of platelet function. *Antioxid Redox Signal.* 2009;11(5):1191-225.
30. Lahav J, Gofer-Dadosh N, Luboshitz J, Hess O, Shaklai M. Protein disulfide isomerase mediates integrin-dependent adhesion. *FEBS Lett.* 2000;475(2):89-92.
31. Markovic I, Stantchev TS, Fields KH, Tiffany LJ, Tomić M, Weiss CD, et al. Thiol/disulfide exchange is a prerequisite for CXCR4-tropic HIV-1 envelope-mediated T-cell fusion during viral entry. *Blood.* 2004;103(5):1586-94.
32. Ryser HJ, Levy EM, Mandel R, DiSciullo GJ. Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. *Proc Natl Acad Sci U S A.* 1994;91(10):4559-63.
33. Wan SW, Lin CF, Lu YT, Lei HY, Anderson R, Lin YS. Endothelial cell surface expression of protein disulfide isomerase activates $\beta 1$ and $\beta 3$ integrins and facilitates dengue virus infection. *J Cell Biochem.* 2012;113(5):1681-91.
34. Tanaka LY, Araújo HA, Hironaka GK, Araujo TL, Takimura CK, Rodriguez AI, et al. Peri/Epicellular Protein Disulfide Isomerase Sustains Vascular Lumen Caliber Through an Anticonstrictive Remodeling Effect. *Hypertension.* 2016;67(3):613-22.
35. Booth C, Koch GL. Perturbation of cellular calcium induces secretion of luminal ER proteins. *Cell.* 1989;59(4):729-37.
36. Willems SH, Tape CJ, Stanley PL, Taylor NA, Mills IG, Neal DE, et al. Thiol isomerases negatively regulate the cellular shedding activity of ADAM17. *Biochem J.* 2010;428(3):439-50.
37. Jordan PA, Gibbins JM. Extracellular disulfide exchange and the regulation of cellular function. *Antioxid Redox Signal.* 2006;8(3-4):312-24.
38. Araujo TLS, Fernandes CG, Laurindo FRM. Golgi-independent routes support protein disulfide isomerase externalization in vascular smooth muscle cells. *Redox Biol.* 2017;12:1004-10.

39. Jasuja R, Furie B, Furie BC. Endothelium-derived but not platelet-derived protein disulfide isomerase is required for thrombus formation in vivo. *Blood*. 2010;116(22):4665-74.
40. Sharda A, Kim SH, Jasuja R, Gopal S, Flaumenhaft R, Furie BC, et al. Defective PDI release from platelets and endothelial cells impairs thrombus formation in Hermansky-Pudlak syndrome. *Blood*. 2015;125(10):1633-42.
41. Crescente M, Pluthero FG, Li L, Lo RW, Walsh TG, Schenk MP, et al. Intracellular Trafficking, Localization, and Mobilization of Platelet-Borne Thiol Isomerases. *Arterioscler Thromb Vasc Biol*. 2016;36(6):1164-73.
42. Swiatkowska M, Szymański J, Padula G, Cierniewski CS. Interaction and functional association of protein disulfide isomerase with alphaVbeta3 integrin on endothelial cells. *FEBS J*. 2008;275(8):1813-23.
43. Popescu NI, Lupu C, Lupu F. Extracellular protein disulfide isomerase regulates coagulation on endothelial cells through modulation of phosphatidylserine exposure. *Blood*. 2010;116(6):993-1001.
44. Tanaka LY, Araujo TLS, Rodriguez AI, Ferraz MS, Pelegati VB, Morais MCC, et al. Peri/epicellular Protein Disulfide Isomerase-A1 Acts as an Upstream Organizer of Cytoskeletal Mechanoadaptation in Vascular Smooth Muscle Cells. *Am J Physiol Heart Circ Physiol*. 2018.
45. Peixoto Á, Geyer RR, Iqbal A, Truzzi DR, Soares Moretti AI, Laurindo FRM, et al. Peroxynitrite preferentially oxidizes the dithiol redox motifs of protein-disulfide isomerase. *J Biol Chem*. 2018;293(4):1450-65.
46. Winterbourn CC, Peskin AV. Kinetic Approaches to Measuring Peroxiredoxin Reactivity. *Mol Cells*. 2016;39(1):26-30.
47. Fernandes DC, Manoel AH, Wosniak J, Laurindo FR. Protein disulfide isomerase overexpression in vascular smooth muscle cells induces spontaneous preemptive NADPH oxidase activation and Nox1 mRNA expression: effects of nitrosothiol exposure. *Arch Biochem Biophys*. 2009;484(2):197-204.
48. Flaumenhaft R, Furie B, Zwicker JJ. Therapeutic implications of protein disulfide isomerase inhibition in thrombotic disease. *Arterioscler Thromb Vasc Biol*. 2015;35(1):16-23.

49. Zhou J, Wu Y, Wang L, Rauova L, Hayes VM, Poncz M, et al. The C-terminal CGHC motif of protein disulfide isomerase supports thrombosis. *J Clin Invest.* 2015;125(12):4391-406.
50. Cho J, Kennedy DR, Lin L, Huang M, Merrill-Skoloff G, Furie BC, et al. Protein disulfide isomerase capture during thrombus formation in vivo depends on the presence of $\beta 3$ integrins. *Blood.* 2012;120(3):647-55.
51. Essex DW, Li M, Miller A, Feinman RD. Protein disulfide isomerase and sulfhydryl-dependent pathways in platelet activation. *Biochemistry.* 2001;40(20):6070-5.
52. Mor-Cohen R. Disulfide Bonds as Regulators of Integrin Function in Thrombosis and Hemostasis. *Antioxid Redox Signal.* 2016;24(1):16-31.
53. Lahav J, Wijnen EM, Hess O, Hamaia SW, Griffiths D, Makris M, et al. Enzymatically catalyzed disulfide exchange is required for platelet adhesion to collagen via integrin $\alpha 2\beta 1$. *Blood.* 2003;102(6):2085-92.
54. Burgess JK, Hotchkiss KA, Suter C, Dudman NP, Szöllösi J, Chesterman CN, et al. Physical proximity and functional association of glycoprotein $Ib\alpha$ and protein-disulfide isomerase on the platelet plasma membrane. *J Biol Chem.* 2000;275(13):9758-66.
55. Hotchkiss KA, Matthias LJ, Hogg PJ. Exposure of the cryptic Arg-Gly-Asp sequence in thrombospondin-1 by protein disulfide isomerase. *Biochim Biophys Acta.* 1998;1388(2):478-88.
56. Huang EM, Detwiler TC, Milev Y, Essex DW. Thiol-disulfide isomerization in thrombospondin: effects of conformation and protein disulfide isomerase. *Blood.* 1997;89(9):3205-12.
57. Essex DW, Miller A, Swiatkowska M, Feinman RD. Protein disulfide isomerase catalyzes the formation of disulfide-linked complexes of vitronectin with thrombin-antithrombin. *Biochemistry.* 1999;38(32):10398-405.
58. Li J, Kim K, Jeong SY, Chiu J, Xiong B, Petukhov PA, et al. Platelet Protein Disulfide Isomerase Promotes Glycoprotein $Ib\alpha$ -Mediated Platelet-Neutrophil Interactions Under Thromboinflammatory Conditions. *Circulation.* 2018.
59. Bowley SR, Fang C, Merrill-Skoloff G, Furie BC, Furie B. Protein disulfide isomerase secretion following vascular injury initiates a regulatory pathway for thrombus formation. *Nat Commun.* 2017;8:14151.

60. Stopa JD, Neuberg D, Puligandla M, Furie B, Flaumenhaft R, Zwicker JI. Protein disulfide isomerase inhibition blocks thrombin generation in humans by interfering with platelet factor V activation. *JCI Insight*. 2017;2(1):e89373.
61. Stopa JD, Baker KM, Grover SP, Flaumenhaft R, Furie B. Kinetic-based trapping by intervening sequence variants of the active sites of protein-disulfide isomerase identifies platelet protein substrates. *J Biol Chem*. 2017;292(22):9063-74.
62. Xu S, Butkevich AN, Yamada R, Zhou Y, Debnath B, Duncan R, et al. Discovery of an orally active small-molecule irreversible inhibitor of protein disulfide isomerase for ovarian cancer treatment. *Proc Natl Acad Sci U S A*. 2012;109(40):16348-53.
63. Xu S, Sankar S, Neamati N. Protein disulfide isomerase: a promising target for cancer therapy. *Drug Discov Today*. 2014;19(3):222-40.
64. Lin L, Gopal S, Sharda A, Passam F, Bowley SR, Stopa J, et al. Quercetin-3-rutinoside Inhibits Protein Disulfide Isomerase by Binding to Its b'x Domain. *J Biol Chem*. 2015;290(39):23543-52.
65. Jasuja R, Passam FH, Kennedy DR, Kim SH, van Hessem L, Lin L, et al. Protein disulfide isomerase inhibitors constitute a new class of antithrombotic agents. *J Clin Invest*. 2012;122(6):2104-13.
66. Kim K, Hahm E, Li J, Holbrook LM, Sasikumar P, Stanley RG, et al. Platelet protein disulfide isomerase is required for thrombus formation but not for hemostasis in mice. *Blood*. 2013;122(6):1052-61.
67. Zwicker JI, Schlechter BL, Stopa JD, Liebman H, Aggarwal A, Puligandla M, et al. Targeting protein disulfide isomerase with the flavonoid isoquercetin to improve hypercoagulability in advanced cancer. *JCI Insight*. 2019.
68. Banfi C, Brioschi M, Wait R, Begum S, Gianazza E, Pirillo A, et al. Proteome of endothelial cell-derived procoagulant microparticles. *Proteomics*. 2005;5(17):4443-55.
69. Fan GQ, Qin RR, Li YH, Song DJ, Chen TS, Zhang W, et al. Endothelial cells microparticle-associated protein disulfide isomerase promotes platelet activation in metabolic syndrome. *Oncotarget*. 2016;7(50):83231-40.
70. Raturi A, Miersch S, Hudson JW, Mutus B. Platelet microparticle-associated protein disulfide isomerase promotes platelet aggregation and inactivates insulin. *Biochim Biophys Acta*. 2008;1778(12):2790-6.

71. Jannes CE, Santos RD, de Souza Silva PR, Turolla L, Gagliardi AC, Marsiglia JD, et al. Familial hypercholesterolemia in Brazil: cascade screening program, clinical and genetic aspects. *Atherosclerosis*. 2015;238(1):101-7.
72. Silva PR, Jannes CE, Marsiglia JD, Krieger JE, Santos RD, Pereira AC. Predictors of cardiovascular events after one year of molecular screening for Familial hypercholesterolemia. *Atherosclerosis*. 2016;250:144-50.
73. Raturi A, Mutus B. Characterization of redox state and reductase activity of protein disulfide isomerase under different redox environments using a sensitive fluorescent assay. *Free Radic Biol Med*. 2007;43(1):62-70.
74. Montano SJ, Lu J, Gustafsson TN, Holmgren A. Activity assays of mammalian thioredoxin and thioredoxin reductase: fluorescent disulfide substrates, mechanisms, and use with tissue samples. *Anal Biochem*. 2014;449:139-46.
75. Foster CK, Thorpe C. Challenges in the evaluation of thiol-reactive inhibitors of human protein disulfide Isomerase. *Free Radic Biol Med*. 2017;108:741-9.
76. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*. 2006;Chapter 3:Unit 3.22.
77. Giannakopoulos B, Gao L, Qi M, Wong JW, Yu DM, Vlachoyiannopoulos PG, et al. Factor XI is a substrate for oxidoreductases: enhanced activation of reduced FXI and its role in antiphospholipid syndrome thrombosis. *J Autoimmun*. 2012;39(3):121-9.
78. Garcia S, Silva-Costa LC, Reis-de-Oliveira G, Guest PC, Baldasso PA, Cassoli JS, et al. Identifying Biomarker Candidates in the Blood Plasma or Serum Proteome. *Adv Exp Med Biol*. 2017;974:193-203.
79. S Cassoli J, Brandão-Teles C, G Santana A, H M F Souza G, Martins-de-Souza D. Ion Mobility-Enhanced Data-Independent Acquisitions Enable a Deep Proteomic Landscape of Oligodendrocytes. *Proteomics*. 2017;17(21).
80. Tang S, Morgan KG, Parker C, Ware JA. Requirement for protein kinase C theta for cell cycle progression and formation of actin stress fibers and filopodia in vascular endothelial cells. *J Biol Chem*. 1997;272(45):28704-11.

81. Prado GN, Romero JR, Rivera A. Endothelin-1 receptor antagonists regulate cell surface-associated protein disulfide isomerase in sickle cell disease. *FASEB J*. 2013;27(11):4619-29.
82. Jones AL, Hulett MD, Parish CR. Histidine-rich glycoprotein: A novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems. *Immunol Cell Biol*. 2005;83(2):106-18.
83. Tsuchida-Straeten N, Ensslen S, Schäfer C, Wöltje M, Denecke B, Moser M, et al. Enhanced blood coagulation and fibrinolysis in mice lacking histidine-rich glycoprotein (HRG). *J Thromb Haemost*. 2005;3(5):865-72.
84. Druhan LJ, Lance A, Li S, Price AE, Emerson JT, Baxter SA, et al. Leucine Rich α -2 Glycoprotein: A Novel Neutrophil Granule Protein and Modulator of Myelopoiesis. *PLoS One*. 2017;12(1):e0170261.
85. Frey EA, Miller DS, Jahr TG, Sundan A, Bazil V, Espevik T, et al. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J Exp Med*. 1992;176(6):1665-71.
86. Essex DW, Li M. A polyclonal antibody to protein disulfide isomerase induces platelet aggregation and secretion. *Thromb Res*. 1999;96(6):445-50.
87. Manickam N, Sun X, Li M, Gazitt Y, Essex DW. Protein disulphide isomerase in platelet function. *Br J Haematol*. 2008;140(2):223-9.
88. Lahav J, Tvito A, Bagoly Z, Dardik R, Inbal A. Factor XIII improves platelet adhesion to fibrinogen by protein disulfide isomerase-mediated activity. *Thromb Res*. 2013;131(4):338-41.
89. Sousa HR, Gaspar RS, Sena EM, da Silva SA, Fontelles JL, AraUjo TL, et al. Novel antiplatelet role for a protein disulfide isomerase-targeted peptide: evidence of covalent binding to the C-terminal CGHC redox motif. *J Thromb Haemost*. 2017;15(4):774-84.
90. Sharda A, Furie B. Regulatory role of thiol isomerases in thrombus formation. *Expert Rev Hematol*. 2018;11(5):437-48.
91. Reinhardt C, von Brühl ML, Manukyan D, Grahl L, Lorenz M, Altmann B, et al. Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. *J Clin Invest*. 2008;118(3):1110-22.

92. Ponamarczuk H, Popielarski M, Stasiak M, Bednarek R, Studzian M, Pulaski L, et al. Contribution of activated beta3 integrin in the PDI release from endothelial cells. *Front Biosci (Landmark Ed)*. 2018;23:1612-27.
93. Hahm E, Li J, Kim K, Huh S, Rogelj S, Cho J. Extracellular protein disulfide isomerase regulates ligand-binding activity of $\alpha\text{M}\beta\text{2}$ integrin and neutrophil recruitment during vascular inflammation. *Blood*. 2013;121(19):3789-800, S1-15.
94. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol*. 2007;7(10):803-15.
95. Sumpio BE, Riley JT, Dardik A. Cells in focus: endothelial cell. *Int J Biochem Cell Biol*. 2002;34(12):1508-12.
96. Badrichani AZ, Stroka DM, Bilbao G, Curiel DT, Bach FH, Ferran C. Bcl-2 and Bcl-XL serve an anti-inflammatory function in endothelial cells through inhibition of NF-kappaB. *J Clin Invest*. 1999;103(4):543-53.
97. Damgaard RB, Gyrd-Hansen M. Inhibitor of apoptosis (IAP) proteins in regulation of inflammation and innate immunity. *Discov Med*. 2011;11(58):221-31.
98. Ganz P, Heidecker B, Hveem K, Jonasson C, Kato S, Segal MR, et al. Development and Validation of a Protein-Based Risk Score for Cardiovascular Outcomes Among Patients With Stable Coronary Heart Disease. *JAMA*. 2016;315(23):2532-41.
99. Hudson DA, Gannon SA, Thorpe C. Oxidative protein folding: from thiol-disulfide exchange reactions to the redox poise of the endoplasmic reticulum. *Free Radic Biol Med*. 2015;80:171-82.
100. Chen K, Detwiler TC, Essex DW. Characterization of protein disulphide isomerase released from activated platelets. *Br J Haematol*. 1995;90(2):425-31.
101. Chen K, Lin Y, Detwiler TC. Protein disulfide isomerase activity is released by activated platelets. *Blood*. 1992;79(9):2226-8.
102. Voigtlaender M, Holstein K, Spath B, Bokemeyer C, Langer F. Expression and release of platelet protein disulphide isomerase in patients with haemophilia A. *Haemophilia*. 2016;22(6):e537-e44.
103. Joshi A, Mayr M. In Aptamers They Trust: The Caveats of the SOMAscan Biomarker Discovery Platform from SomaLogic. *Circulation*. 2018;138(22):2482-5.

104. Chang NS, Leu RW, Rummage JA, Anderson JK, Mole JE. Regulation of macrophage Fc receptor expression and phagocytosis by histidine-rich glycoprotein. *Immunology*. 1992;77(4):532-8.
105. Olsen HM, Parish CR, Altin JG. Histidine-rich glycoprotein binding to T-cell lines and its effect on T-cell substratum adhesion is strongly potentiated by zinc. *Immunology*. 1996;88(2):198-206.
106. Behr Andersen C, Lindholt JS, Urbonavicius S, Halekoh U, Jensen PS, Stubbe J, et al. Abdominal Aortic Aneurysms Growth Is Associated With High Concentrations of Plasma Proteins in the Intraluminal Thrombus and Diseased Arterial Tissue. *Arterioscler Thromb Vasc Biol*. 2018;38(9):2254-67.
107. Hayashida K, ., Bagchi A, Miyazaki Y, Hirai S, Seth D, M.G. S, et al. Improvement in outcomes after cardiac arrest and resuscitation by inhibition of s-nitrosoglutathione reductase.: *Circulation*; 2018.
108. Han J, Weisbrod RM, Shao D, Watanabe Y, Yin X, Bachschmid MM, et al. The redox mechanism for vascular barrier dysfunction associated with metabolic disorders: Glutathionylation of Rac1 in endothelial cells. *Redox Biol*. 2016;9:306-19.
109. Végvári A, Welinder C, Lindberg H, Fehniger TE, Marko-Varga G. Biobank resources for future patient care: developments, principles and concepts. *J Clin Bioinforma*. 2011;1(1):24.

9. Apêndice

Apêndice A

Artigo 1: *Protein Disulfide Isomerase Plasma Levels in Healthy Humans Reveal Proteomic Signatures Involved in Contrasting Endothelial Phenotypes. Redox Biology. 2019; in press.*

Protein Disulfide Isomerase Plasma Levels in Healthy Humans Reveal Proteomic Signatures Involved in Contrasting Endothelial Phenotypes

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Abbreviations: CM: conditioned medium, EC: endothelial cell, ELISA: enzyme-linked immunosorbent assay, ER: endoplasmic reticulum, ERp57: endoplasmic reticulum protein 57, ERp72: endoplasmic reticulum protein 72, FBS: fetal bovine serum, GSNO: S-nitrosoglutathione, GSSG: glutathione disulfide, HRG: histidine-rich glycoprotein, HUVEC: human umbilical vein endothelial cell line, MPB: 3-(N-maleimido-propionyl) biocytin, PDI: protein disulfide isomerase, pecPDI: peri/epicellular PDI, PRP: Platelet-rich plasma, TMB: tetramethylbenzidine.

ABSTRACT

Redox-related plasma proteins are candidate reporters of protein signatures associated with endothelial structure/function. Thiol-proteins from protein disulfide isomerase (PDI) family are unexplored in this context. Here, we investigate the occurrence and physiological significance of a circulating pool of PDI in healthy humans. We validated an assay for detecting PDI in plasma of healthy individuals. Our results indicate high inter-individual (median= 330 pg/ml) but low intra-individual variability over time and repeated measurements. Remarkably, plasma PDI levels could discriminate between distinct plasma proteome signatures, with PDI-rich (>median) plasma differentially expressing proteins related to cell differentiation, protein processing, housekeeping functions and others, while PDI-poor plasma differentially displayed proteins associated with coagulation, inflammatory responses and immunoactivation. Platelet function was similar among individuals with PDI-rich vs. PDI-poor plasma. Remarkably, such protein signatures closely correlated with endothelial function and phenotype, since cultured endothelial cells incubated with PDI-poor or PDI-rich plasma recapitulated gene expression and secretome patterns in line with their corresponding plasma signatures. Furthermore, such signatures translated into functional responses, with PDI-poor plasma promoting impairment of endothelial adhesion to fibronectin and a disturbed pattern of wound-associated migration and recovery area. Patients with cardiovascular events had lower PDI levels vs. healthy individuals. This is the first study describing PDI levels as reporters of specific plasma proteome signatures directly promoting contrasting endothelial phenotypes and functional responses.

Keywords: protein disulfide isomerase, plasma proteome, endothelial cells, plasma protein signatures, thiol proteins

INTRODUCTION

Redox processes play major integrative roles in plasma¹ and endothelial (patho)physiology. Redox-related plasma proteins are thus candidate reporters of vascular structure and function. In this context, thiol proteins from the protein disulfide isomerase (PDI) family remain unexplored². The family prototype is PDIA1 (henceforth designated as PDI), a 55 kDa thiol oxidoreductase chaperone mainly from the endoplasmic reticulum (ER), which depicts a U-shaped structure comprising 4 thioredoxin tandem domains termed *a-b-b'-a'*, plus a C-terminal domain. The *a*-type domains at each arm of the "U" contain catalytic active sites carrying Cys-Gly-His-Cys motifs, which enable PDI to undergo reduction-oxidation cycles^{3,4}. The *b*-type domains at the bottom of the "U" are enriched in hydrophobic residues and involved in substrate binding. PDI also displays chaperone activity, *per se* thiol redox-independent, although enhanced upon oxidation^{3,5}. Canonical PDI functions consist of introduction or isomerization of disulfide bonds in nascent ER proteins, as well as their retrograde cytoplasmic transport during ER-associated protein degradation³. Despite being primarily an ER protein, PDI has been reported at other intracellular locations and also at the cell surface and extracellular milieu (a pool we have termed peri/epicellular PDI=*pecPDI*)^{6,7}.

A *pecPDI* pool released from activated endothelial cells (EC) and platelets exerts critical roles in thrombus formation after vascular injury. PDI inhibition strongly inhibits both platelet accumulation and fibrin generation at the injury site⁸. The *a'* rather than *a*-domain PDI cysteines are specifically involved in thrombosis and platelet activation⁹. In EC, *pecPDI* is estimated as <2% of cellular PDI and its externalization occurs without detectable plasma membrane damage via yet unclear secretion routes that are mostly Golgi-independent¹⁰. However, PDI extravasation substantially increases upon cell injury, given the very high intracellular expression of PDIs in general¹⁰. In EC, PDI is detectable in vesicles distinct from those containing Von Willebrand factor¹¹, while impairment of lysosome and dense granule biogenesis inhibits EC or platelet PDI secretion and enhances bleeding¹². In platelets, PDI is located at the dense tubular system and likely exocytosed upon activation¹³. The roles of PDIs in thrombosis are at the forefront of translational advances in novel antithrombotic agents⁸, with the list of new PDI inhibitors growing quite fast recently¹⁴⁻¹⁶. A multi-center efficacy clinical trial of isoquercetin as PDI inhibitor against cancer-associated thrombosis is currently under way¹⁷.

Thrombosis-related extracellular PDI substrates have been actively investigated and include several integrins, which were well documented in platelets, EC and vessel wall¹⁸. The main effect of PDI (as well as ERp57 and ERp72) is to reduce integrin disulfide bonds to support transition from extended-moderate to extended-high affinity conformation^{19, 20}, although our group reported *pecPDI*-mediated $\alpha 5$ -integrin oxidation upon short-term EC mechanostimulation¹⁰. While PDI has no membrane-binding or transmembrane motifs, binding to integrins (e.g., $\beta 3$) accounts for extracellular PDI retention²¹. In addition to integrins, kinetic trapping studies identified multiple PDI substrates in stimulated platelet-rich plasma, including vitronectin, complement factors 3 and 5, C4b-binding protein, histidine-rich glycoprotein (HRG), thrombospondin 1, coagulation factor V, serpin B6, glutaredoxin-1²²⁻²⁴. Importantly, peculiar redox properties of PDI likely modulate the mode of PDI-substrate interaction. The slow reaction rate with hydrogen peroxide²⁵ indicates that PDI is unlikely to act as a mass redox sensor such as peroxiredoxins⁷. Also, the low redox potential of N-terminal PDI domain Cys may support PDI oxidative capacity over a larger range of ambient

reducing potentials than, e.g., thioredoxin²⁶. Moreover, PDI supports protein complex organization, such as for NADPH oxidases²⁷. These properties bring two implications. First, PDI likely promotes targeted rather than nonspecific redox modulation of its substrates⁷. Second, in the extracellular environment, in which PDI concentration is lower than that of its substrates - contrarily to the ER lumen - the amount of free PDI can be affected by type and binding capacity of substrates. Thus, PDI levels could potentially act as reporter of distinct proteome signatures in extracellular compartments such as plasma.

However, there is little information on circulating plasma levels of vascular thiol isomerases. One study reported a low-level pool of PDI in plasma from healthy humans²⁸ and some reports showed PDI release from platelets^{29,30} or associated with EC and platelet microparticles³¹⁻³³. In contrast, it has been recently suggested that extracellular PDI and other thiol isomerases are absent from the circulation as a way to suppress thrombus formation²², while upon vascular wall injury PDI release from disrupted endothelium and bound platelets relieves such suppression and promotes thrombosis via disulfide modification of covalently-bound substrates. Here, we addressed the occurrence and physiological significance of a circulating pool of PDI in healthy humans, with a focus on its role as reporter of distinct plasma protein signatures involved in endothelial phenotype and functional responses potentially associated with disease risk.

MATERIALS AND METHODS

Study Population and Ethics Approval

We selected 35 healthy volunteers with characteristics shown in Table 1. All participants had no prior history of chronic or acute illness including cardiovascular, digestive, metabolic or tumoral conditions. None of them made use of tobacco or other drugs, as well as chronic medications. The study protocol was approved by the Institutional Scientific/Ethics Committee (CAPPesq number 3334/09/085) and written informed consent was obtained from all participants prior to entering the study.

Measurement of PDI by ELISA

Soluble PDI antigen was measured using the Human P4HB Pair Set enzyme-linked immunosorbent assay (ELISA) (SinoBiological-SEK10827, Lot numbers KW05NO2203 and KW09NO1102) according to the manufacturer's protocol with minor modifications. Briefly, 96-well microplates were coated with 100 μ l/well of diluted capture antibody and incubated overnight at 4°C, followed by addition of each sample (100 μ L) for 2h at room temperature (RT) with agitation. Thereafter, 100 μ l of detection antibody conjugated to horseradish-peroxidase (HRP) was incubated for 2h at room temperature (RT) with agitation. Plates were washed three times with phosphate buffered saline (PBS)-tween/20 (0.5%) after each incubation. Finally, 200 μ l of tetramethylbenzidine (TMB) solution were added for 30 min with agitation. The optical density of each well was determined immediately using a microplate reader (SpectraMax-340, Molecular Devices) set to 450 nm. Values were determined according to a standard curve (log/log curve-fit). Platelet-poor plasma (no dilution), recombinant PDI solutions (reduced, oxidized, S-nitrosylated),

human cell lysates, conditioned medium, and recombinant Erp57 (OriGene-TP305940) and ERp5 (OriGene-TP301710) were analyzed.

PDI purification

Histidine-tagged full length human PDI was cloned into the pET28a vector (Novagen) and overexpressed in *E. coli* strain BL21 (DE3) codon plus. PDI was first isolated and purified with an immobilized metal affinity resin as previously described³⁴.

Preparation of reduced, oxidized and S-nitrosylated PDI

PDI was reduced with 10 mM of GSH (Sigma) or oxidized with 10 mM of diamide (Sigma) overnight at 4°C. For preparing the nitrosylated protein, PDI was incubated with 200-fold molar excess of S-nitrosoglutathione (GSNO) (Sigma) for 30 min at 37°C. Samples were then run through a desalting column (Microcon YM-50, Amicon) to remove excess products. The concentrations of PDI in solutions were assessed at 280 nm ($\epsilon = 45,565 \text{ M}^{-1} \text{ cm}^{-1}$).

Western blot analysis

Equal amounts of protein from HUVEC, HCT116 and HKE3 lysates or conditioned medium (CM) were resolved by SDS-PAGE (12% gel density). The following primary antibodies used were anti-PDI (capture antibody from Human P4HB Pair Set ELISA kit or clone RL90, Thermo-Fisher), anti-ERp72 (ab82587, Abcam), anti-ERp57 (ADI-SPA-585, Enzo). Fluorescence-coupled secondary antibody was purchased from Odyssey and fluorescent immunoblottings were scanned with the Odyssey near-infrared imaging system (Li-cor).

Preparation of platelet-poor plasma

Blood for platelet-poor plasma samples were collected by venipuncture into EDTA tubes and centrifuged for 15 min at $2.500\times g$. After centrifugation the plasma phase was used for further analysis or for stored at -80°C .

Immunoprecipitation

Fresh platelet-poor plasma samples pooled from 3 donors (10 mL) was diluted 1:1 in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton-X 100) supplemented with protease (1 mM PMSF, 1 $\mu\text{g}/\text{mL}$ leupeptin and aprotinin) and phosphatase inhibitors (50 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate) and 10 mM MgCl_2 . The plasma sample was incubated overnight at 4°C under agitation with anti-PDI antibody (10 μg , rabbit EnzoLife, SPA890) followed by incubation with 50 μl Protein G-coated magnetic beads (GE HealthCare) for 4h at 4°C. Beads were successively washed in lysis buffer to remove contaminating material. PDI was detected by reducing SDS-PAGE and immunoblotted with a mouse monoclonal antibody (clone RL90, Thermo-Fisher). Blots were scanned with the Odyssey near-infrared

fluorescence imaging system (Li-cor). Results are representative from at least 3 independent experiments.

Measurement of PDI reductase activity

The probe di-eosin-glutathione disulfide (GSSG) was prepared through the incubation of 200 μM GSSG (Sigma) with 2 mM eosin-isothiocyanate (Thermo-Fisher) in 0.1 M potassium phosphate (PE) containing 2 mM EDTA (pH 8.5) at RT, overnight in the dark³⁵. The mixture was run through a PD-G25 (GE HealthCare) column and different fractions were collected. A fold-change of fluorescence (Exc: 520 nm, Emis: 545 nm) was calculated using samples subjected to either buffer or 20 mM DTT. Any fraction with a fold-change >5 was kept. The concentration of di-eosin-GSSG was determined by using the molar absorption coefficient for eosin ($\epsilon = 56,000 \text{ M}^{-1} \text{ cm}^{-1}$) at 525 nm in PE buffer³⁶. Reduction of plasma was carried out by incubation of 0.5 mL of fresh platelet-poor plasma in assay buffer (PE pH: 7.4, containing 2 mM EDTA), 5 μM DTT and 150 nM of di-eosin-GSSG in the presence or absence of 60 μM of rutin (quercetin-3-rutinoside) (Sigma). The increase in fluorescence was determined for 20 min by excitation at 520 nm and emission at 545 nm in a SpectraMax-M5. The reduction of 150 nM di-eosin-GSSG by 5 μM DTT served as a negative control. Five independent experiments were performed, and each sample was detected in duplicate. Data were normalized to the fluorescence of total plasma (control) and represented as percent reductase activity.

Separation of microparticle fraction

Protocol was performed as described previously by³⁷. Briefly, platelet-poor plasma (5 mL) was diluted with an equal volume of PBS and centrifuged for 30 min at $2,000\times g$, 4°C . Supernatants were transferred to ultracentrifuge tubes and centrifuged for 45 min at $12,000\times g$, 4°C . Subsequently, supernatants were submitted to centrifugation for 2h at $110,000\times g$, 4°C . After that, pellets were re-suspended in 1 mL PBS, filtered through a $0.22\text{-}\mu\text{m}$ filter and again centrifuged for 70 min at $110,000\times g$, 4°C (twice). The final pellets were suspended in 100 μL PBS, lysed in lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 150 mM NaCl) with protease inhibitors (1 mM PMSF, 1 $\mu\text{g}/\text{mL}$ leupeptin and aprotinin) and stored at -80°C .

EZ-Link Sulfo-NHS-Biotin and MPB-labelled assay for plasma PDI redox state

To assess the redox state of plasma PDI, 200 μL of fresh platelet-poor plasma aliquots were separately labelled by a large excess of MPB [3-(N-maleimido-propionyl) biocytin] (4 mM, Molecular Probes) or EZ-Link Sulfo-NHS-(N-hydroxysuccinimido)-Biotin (4 mM, Thermo-Scientific) in the dark, under agitation at RT for 30 min. All reagents were prepared in 20 mM HEPES buffer containing 0.14 M NaCl, 1.5 mM CaCl_2 , 4 mM KCl, 0.5 mM Na_2HPO_4 , at pH 7.4 (HBS) as described³⁸. Thereafter, samples were diluted in the same buffer (1 mL) and incubated for additional 10 min, in the dark at RT. Unbound EZ-Link Sulfo-NHS-Biotin or MPB was then removed by acetone precipitation. The protein pellets were then re-suspended in 250 μL of PBS-Tween/20 (0.05%) and constitute the labelled plasma samples. Additionally, unlabelled plasma

samples were also incubated only with HBS (without MPB or EZ-Link Sulfo-NHS-Biotin) as a control. As an internal control for variations in sample labelling with MPB and EZ-Link Sulfo-NHS-Biotin, a standard in-house pool of plasmas was used in each assay. All samples and controls were run concomitantly in parallel. To perform the ELISA for PDI determination, first 96-well plates were coated with anti-PDI capture antibody from Human P4HB Pair Set ELISA kit. Before adding labelled samples, PDI plates were pre-washed three times with PBS-tween/20 (0.5%) and blocked for 1h with 2% BSA/PBS-tween. Labelled samples were then added to the 96-well plates (100 μ L/well) in duplicate and incubated at RT for 2h with agitation. After washing three times with PBS-tween, a mouse monoclonal anti-biotin (1:200, peroxidase-conjugate) (Clone BN-34, Sigma) diluted in 0.5% BSA/PBS-tween/20 was added (100 μ L/well) and incubated at RT for 2h with agitation. At the final step, wells were washed three times with PBS-tween, 200 μ l of TMB solution was added for 30 min with agitation and samples were read at OD 450 nm (SpectraMax-340, Molecular Devices). Amine-labelled PDI sample was considered as total PDI, while MPB-labelled PDI sample was considered reduced PDI.

Cell culture

A selection-immortalized human umbilical vein endothelial cell line (HUVEC line, from ATCC)^{10, 39, 40} was maintained in RPMI (GIBCO Cell Culture systems, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, 25 mg/mL penicillin and 10 mM HEPES at 37°C in 95% O₂/5% CO₂. Human colon carcinoma cell lines HCT116 and HKE3 were a kind gift from Dr. Walter Kolch (University College Dublin, Belfield, Dublin 4, Ireland). HCT116 and HKE3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (GIBCO Cell Culture systems, Invitrogen) at 37°C in 95% O₂/5% CO₂.

EC plasma incubation

Adhering confluent HUVEC cells were harvested and seeded at 2.5×10^6 cells in plastic petri dishes (100 mm) with a final volume of 10 mL culture medium (containing 10% FBS) per dish. After culturing for 22–24h, cells were washed with PBS (pH: 7.4) and cultured for additional 24h in medium in which FBS (5%) was replaced with 5% of either PDI-poor or PDI-rich plasma obtained from a pool of equal volumes of 3 individual samples. The 5% concentration was chosen from validation experiments; incubation with 10% human plasma associated with significant EC detachment, although they remained viable during the 24-h period.

Cell viability assessment

After 22–24h in culture conditions, HUVEC were washed with PBS (pH 7.4) and cultured for 24h in medium in which FBS was substituted for 5% of platelet-free plasma pools from 3 individuals with PDI-poor or PDI-rich plasma. After that, cells were mixed with Trypan Blue solution (0.4%). Colored (non-viable) and dye-excluding (viable) cells were counted on automated cell counter (Countess II FL, Thermo-Scientific).

Proteomic analysis

Sample preparation and UPLC-HDMSE analysis

Blood plasma sample preparation

Forty microliters of blood plasma from 6 healthy donors were diluted in 120 μL of buffer solution A, from the MARS Hu14 column manufacturer's protocol (Agilent) to remove the highest abundant proteins. The immunoaffinity chromatography was performed according to⁴¹.

Conditioned medium (CM) from HUVEC and sample preparation

HUVEC were cultured and treated as describe above ('EC plasma incubation' section). To obtain the CM, cell culture media were removed, carefully washed with PBS and each CM sample was obtained following incubation with 6 mL of serum-free medium. After 8h, supernatants were collected and depleted of cell debris by centrifugation (1,400 rpm, 10 min, 4°C), followed by medium centrifugation and 3-fold concentration, using SpeedVac. The concentrated medium was stored at -80°C for additional analysis. Protein concentrations were determined by Bradford assay and protein samples (50 μg each sample) were transferred into SDS-loading buffer (156.25 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 4% (v/v) glycerol, 0.5% (v/v) 2-mercaptoethanol), boiled (5 min, 95°C) and then subjected to SDS-PAGE (12% gel density). Each lane was divided into >20 fragments. The proteins within the gel fragments were in-gel-trypsinized at 37°C overnight. To extract the peptides, 50 mM $(\text{NH}_4)_2\text{CO}_3$ (50 μL) and 2 \times 50 μL 50% (v/v) acetonitrile, 5% (v/v) formic acid were added successively to the gel pieces. The volume of the extraction solution was reduced to <20 μL using SpeedVac.

Mass spectrometry acquisition

Peptides (0.5 μg) were automatically loaded into a high-throughput sample manager (SM) in a UPLC M-Class System (Waters Corporation, Milford, MA), coupled online to a Synapt G2-Si mass spectrometer (Waters Corporation, Milford, MA). Trapped peptides were eluted with an acetonitrile gradient from 7% to 40% (v/v) for 52 min at a flow rate of 0.5 $\mu\text{L}/\text{min}$ and analyzed directly into a Synapt G2-Si. Peptides were ionized using a nano-electrospray source in positive mode. Results were obtained in a data-independent mode (DIA) combined with ion mobility acquisition mode (HDMSe). The mass spectrometer parameters were set according to³⁹.

Protein identification and quantitation

Proteins were identified and quantified by using dedicated algorithms of the Progenesis QI for Proteomics software (Waters Corporation). Data were searched against the UniProt human proteomic database: for blood plasma, we used the complete database (version: 2016_06) and for HUVEC cells we employed the reviewed database (version: 2017_10). The following parameters were considered to identify peptides in blood plasma samples: 1) Digestion by trypsin with at most one missed cleavage; 2) variable modifications by oxidation (M) and fixed modification by carbamidomethyl (C); 3) calculated false discovery rate (FDR) less than 4% across all conditions and replicates; 4) mass error less than 20 ppm. One or more ion fragments per peptide, three or more fragments per protein and one unique or more peptides per protein were required for ion

matching. The following parameters were considered to identify peptides in HUVEC samples: 1) Digestion by trypsin with at most one missed cleavage; 2) variable modifications by oxidation (M) and fixed modification by carbamidomethyl (C); 3) calculated false discovery rate (FDR) less than 1% across all conditions and replicates; 4) mass error less than 20 ppm. Two or more ion fragments per peptide, five or more fragments per protein and one unique or more peptides per protein were required for ion matching. Identifications that did not satisfy these criteria were rejected. The final, confirmed list of proteins only considered proteins identified by at least 2 peptides. Also, differences between groups were analyzed using one-way ANOVA and only a p-value < 0.05 was considered significant. Keratin proteins were excluded from our data.

Enrichment analysis

Metacore software (Thomson Reuters, MI, USA) was used to perform functional enrichment analysis and to create biological protein networks. Enriched pathways were considered significant at p value <0.05.

Platelet aggregation

Platelet-rich plasma (PRP) samples were obtained from individuals who had not used antiplatelet medications for at least 10 days prior to venipuncture. Blood samples were anticoagulated with trisodium citrate 3.2% (blood/anticoagulant 9:1 v/v) and centrifuged at 190×g for 15 min to obtain PRP. PRP ($2-3 \times 10^8/\text{mL}^{-1}$) were incubated with ADP (10 μM), collagen (5 $\mu\text{g}/\text{mL}$) or calcium ionophore-A23187 (20.5 μM) for 5 min and then aggregation was measured using a whole blood/lumi-aggregometer (Chronolog, 560VS). Whole blood samples were anticoagulated with trisodium citrate 3.2% (blood/anticoagulant 9:1 v/v) and thereafter incubated with collagen (25 μM) for 10 min. Aggregation was measured as describe for PRP.

RT² Profiler PCR array

HUVEC were cultured and prepared as described in 'EC plasma incubation' section. After that, 2×10^6 cells were collected, centrifuged and homogenized in Trizol (Invitrogen) for RNA extraction. The quantity and quality of RNA samples were determined using Nanodrop ND2000 (Thermo-Fisher). RNA samples (1.5 μg) were reverse-transcribed to synthesize cDNA library using RT² First Strand Kit (Qiagen). A cDNA library was used as the template for the subsequent PCR analysis in a StepOnePlus™ Real-Time PCR System. The expression levels of 84 genes (listed in Supplementary Table 3) were analyzed using RT² Profile PCR array (PAHS-038, Qiagen). The PCR array experiment was performed from 3 independent experiments (each one performed in triplicate). Relative quantities of mRNA were calculated using $\Delta\Delta\text{Ct}$ with β -2-microglobulin as the reference.

Cell adhesion assay

Flat bottom 96-well plates were coated with either fibronectin (Sigma) (5 µg/mL) or collagen type-I (Sigma) (10 µg/mL) overnight at 4°C and then blocked for 2h with 1% BSA/PBS. Cultured HUVEC prepared as described in 'EC plasma incubation' section were washed with PBS and detached with PBS-EDTA (500 µM, pH 7.4). After that, cells were re-suspended in RPMI containing 0.2% BSA (adhesion buffer), added to coated wells at a volume of 150 µL (4×10⁴ cells/well) and allowed to adhere to the substrate for 1h (fibronectin) or 4h30min (collagen) at 37°C. Unattached cells were removed by gently washing three times with PBS. Residual attached cells were fixed by 2% glutaraldehyde for 30 min at RT, and then plates were washed three times with PBS and stained by 0.1% crystal violet (30 min at RT), followed by lysis with 1% SDS. Absorbance was then measured at 600 nm in a microplate reader (SpectraMax-340, Molecular Devices).

Wound healing assay

HUVEC migration was investigated by wound healing assay as described previously⁴². Cells were seeded (2×10⁵) in flat bottom 24-well plates. After 48h, confluent cell monolayers were incubated for 5 min with HBSS (Hanks' Balanced Salt solution) without Ca²⁺ and Mg²⁺ (H6648, Sigma). Thereafter, a wound was created by scratching the cells once with a sterile tip (500 µm in diameter). The cultures were washed twice with HBSS to remove cell debris. HUVEC were then incubated with RPMI alone or supplemented with 5% of either FBS, PDI-poor plasma or PDI-rich plasma. Cells were then transferred to a microscope chamber at 37°C with 95% O₂/5% CO₂ (Axiovert 200, Zeiss) and pictures were taken at 0h, 8h, 16h and 24h (3 separate fields/well) with 5x objective. Covered surface areas were measured using Image-J software.

Statistical Analysis

Normally and non-normally distributed data were presented as mean ± standard error of the mean (SEM) and median with interquartile ranges, respectively. Differences between two groups were analyzed using paired Student's t-test to compare two groups or one-way ANOVA plus Student-Tukey's test or the Mann-Whitney test. Correlation coefficients were according to the method of Spearman. A p-value <0.05 was considered significant. Analyses were carried out using GraphPad Prism v.7. Linear regression was used to estimate the association between plasma PDI and traditional risk factors in patients from databank. All analyses were performed using at least three independent experiments repeated in distinct times, with 2 or 3 replicates for each measurement, as specified. Normality tests (Shapiro-Wilk and D'Agostino-Pearson Omnibus) were performed followed by frequency distribution of PDI levels using Prisma parameters.

RESULTS

ELISA assay validation for plasma PDI

Circulating plasma PDI levels were assessed by enzyme-linked immunosorbent assay (ELISA). After testing distinct approaches, which proved to be insufficiently sensitive, we chose a commercially available kit not yet validated in the literature (Human P4HB Pair Set, SinoBiological), which was able to detect levels as low as 47 pg/ml (Suppl.Fig.S1-A and B). Assay curves with recombinant PDI confirmed its accurate detection (Fig.1A), while the assay was equally able to detect reduced, oxidized or S-nitrosylated PDI – Suppl.Fig.S1-C). Additionally, we quantified intracellular PDI from human cell lysates (Fig.1B) in parallel with western analysis using ELISA's capture antibody in the same samples, showing immunostaining of a single band consistent with PDI (Fig.1C). We also tested possible cross-reactivity with other PDIs using recombinant PDI, ERp57 and ERp5 and showed that this assay recognized only PDI (Fig.1D). Detection of circulating PDI was additionally confirmed by immunoprecipitation (Fig.1E, Suppl.Fig.S1-D). Also, to assess whether circulating PDI is active, we measured the quercetin-3-rutinoside (rutin)-inhibitable fraction of total di-eosin-GSSG reductase activity; the use of rutin, a well-reported PDI inhibitor⁴³, enhances the specificity of this otherwise nonspecific reductase assay. Rutin inhibited di-eosin-GSSG turnover by *ca.*70% and 45% in PDI-rich and PDI-poor plasma, respectively (Fig.1F and Suppl.Fig.S1-E). A conceptually analogous approach allowed evidence of plasma PDI activity in sickle-cell disease transgenic mice⁴⁴. These results support a plasma pool of active PDI in healthy individuals.

Plasma levels of PDI show high inter-individual but low intra-individual variability

The amount of PDI detected by our assay was low, at pM levels, and their distribution was not normal, as confirmed by specific tests (Fig.2A–B). Microparticle-associated PDI accounted for a minor fraction of total PDI levels in our population (Suppl.Fig.S2-A). Plasma PDI concentrations depicted high inter-individual variability, with values running from undetectable/very low until >1000 pg/mL (~18 pM). As the median value was 330 pg/mL (~6 pM) (Fig.2A), we designated values ≤ 330 pg/mL as PDI-poor plasma, as opposed to PDI-rich plasma (>330 pg/mL). Remarkably, opposite to inter-individual variability, the intra-individual variability was quite low, so that values assessed under distinct conditions over time were close and reproducible (Fig.2C). For that, plasma was collected 10-15 times, spaced from days to weeks over a 10-15 month span and circulating PDI levels exhibited in most cases a small range of values (Fig.2C). Also, in a cohort of 5 individuals, PDI levels were serially collected 3 times over a 9-h period; again, variability among results was negligible (Suppl.Fig.S2-D). Importantly, we assessed the possible influence of variables reflecting traditional cardiovascular and inflammation risk factors on PDI levels. There was no significant correlation between PDI plasma levels with age, triglyceride levels, high-density lipoprotein (HDL) cholesterol levels, C-reactive protein (CRP) values, white and red blood cell counts and platelet counts, while there was a non-robust direct correlation with total and LDL cholesterol ($p < 0.05$) (Suppl.Fig.S3).

Thiol redox state of plasma PDI pool

PDI exhibits a pool of distinct redox states: fully/partially reduced, fully/partially oxidized to intramolecular disulfides, oxidized to intermolecular disulfides (e.g., S-glutathionylated) or S-nitrosylated. We developed assays with biotinylated probes (see Materials and Methods) using a pooled (3 individuals) *in-house* plasma sample as internal reference control for their labeling amount (Suppl.Fig.S2-C). Our results showed that the majority (60-80%) of plasma PDI was in the reduced state (Fig.2D and Suppl.Fig.S2-B), with no difference in PDI-poor vs. PDI-rich plasma.

Distinct PDI concentrations uncover contrasting plasma protein signature patterns

Given the low intra-individual variability despite widely distinct inter-individual plasma levels, we hypothesized that PDI concentrations could behave as a window to reveal individual plasma protein signatures. Thus, we performed quantitative proteome analyses comparing PDI-poor vs. PDI-rich plasma. Plasma samples were depleted of top 14 high-abundant proteins, as these can mask low-abundance protein detection given the huge concentration dynamic range. Low abundant proteins were analyzed by shotgun mass spectrometry-based proteomics, allowing identification of 1,788 proteins. Quantitative analysis indicated 125 upregulated proteins differentially expressed between PDI-poor and PDI-rich plasma (65 and 60, respectively) (Suppl.Tables S1–2). To investigate whether these proteins compose signatures for specific processes, we used Metacore software database. Importantly, the protein subset upregulated in PDI-poor plasma was preferentially associated with immuno-inflammation, coagulation and platelet interactions, with 50% pathways and 70% biological processes including blood coagulation and immune response. Main examples included: coagulation factors XII and XI and kininogen 1, from the intrinsic coagulation pathway; proteins associated with plasminogen and fibrinogen interactions; proteins involved in platelet interactions, such as histidine-rich glycoprotein (HRG)^{45, 46}; immune-related proteins, such as leucine-rich alpha-2-glycoprotein⁴⁷ and CD14⁴⁸. In particular, several complement-related proteins were preferentially identified in PDI-poor plasma. Biological processes/pathways and main gene ontology processes identified in the biological networks are depicted in Fig.3 (left diagram) and Suppl.Fig.S4-A. In contrast, the subset of proteins preferentially upregulated in PDI-rich plasma (vs. PDI-poor plasma) was related to cell adhesion (e.g. cadherin-5), cytoskeleton (e.g. myosin-8) and cell cycle (e.g. MAD2-like protein 1), as depicted in Fig.3 (right diagram) and Suppl.Fig.S4-B. These results suggest that PDI levels can reflect distinct processes and gene ontology profiles of proteomic signatures in the plasma of healthy individuals with no other obvious differences in their conventional risk-associated indicators.

PDI level-associated protein signatures did not correlate with platelet aggregation patterns

We next interrogated whether the observed differences in PDI plasma levels associate with different platelet function profiles. Platelet aggregation assays were performed in platelet-rich plasma collected from individuals with PDI-poor vs. PDI-rich plasma stimulated for 5 min with ADP (10 μ M), collagen (5 μ g/mL) or calcium ionophore A23187 (20.5 μ M). Platelet aggregation responses to each of these agonists showed no differences between PDI-poor vs. PDI-rich plasma,

all of them having on average ca.80% extent of aggregation (Fig.4A–B). Similar results were observed for whole-blood platelet aggregation (Fig.4C–D). Since PDI is secreted after platelet activation, we next investigated soluble PDI levels after platelet aggregation. Interestingly, PDI levels in platelet-poor plasma were decreased post-aggregation in most samples regardless of the agonist, with few exceptions (Fig.4E). Both effects were unrelated to circulating PDI levels or degree of platelet activation.

Distinct PDI levels in plasma recapitulate contrasting endothelial gene expression and secretome patterns

Since EC can secrete PDI^{49,10} and *pec*PDI modulates several EC effects, we assessed effects of incubation of PDI-poor or PDI-rich plasma in cultured EC. Cultured human umbilical vein EC (HUVEC) were incubated for 24h in medium with standard FBS (5%) or in medium in which FBS was replaced with 5% plasma collected from individuals with low or high PDI levels, and the patterns of EC gene expression and protein secretion were assessed. Gene expression changes were analyzed by PCR array covering 84 prespecified genes (Suppl.Table S3) involved in blood coagulation, cell adhesion, lipid transport/metabolism, chemotaxis/inflammation, cell growth/proliferation and apoptosis. Plasma-exposed EC exhibited a fibroblast-like rather than their normal cobblestone-like phenotype (Suppl.Fig.S5-A), consistent with EC activation, i.e., switch from baseline to a state primed for thrombosis, proliferation and vasoconstriction^{50, 51}. While shape changes were indistinguishable following incubation with PDI-poor or PDI-rich plasma, EC gene expression responses were significantly different for each type of plasma vs. FBS-controls, suggesting distinct EC activation patterns (Fig.5A). Overall, 48% of genes were simultaneously upregulated with PDI-poor and downregulated with PDI-rich plasma (Fig.5B). Main examples include integrins involved in leukocyte-EC adhesion: integrin ITGAL/ITGB2 (ITGB2), α X β 2 (ITGAX), intracellular adhesion molecule 1 (ICAM1), selectins E (SELE) and L (SELL), as well as coagulation-related genes such as von Willebrand factor (VWF), fibrinogen α -chain (FGA) and fibrinolysis inhibitors such as plasminogen-activator inhibitors 1(SERPINE1) and 2(SERPINB2). Identical patterns occurred for 10/17 inflammatory/chemotaxis genes and 6/14 lipid transport/metabolism genes, with the other showing diverse behaviors (Fig.5B–C). In contrast, there was no clear pattern for growth/proliferation genes (Fig.5B–C). Among 9 apoptosis-related genes, 5 were upregulated with PDI-poor and downregulated with PDI-rich plasma (FAS, BCL2L1, BCL2A1, BID, BAX), while 4 were upregulated in both cases. Anti-apoptotic genes BCL2 and BIRC3, which also regulate inflammatory signaling and immunity^{52, 53}, underwent the highest fold-changes vs. controls, accentuated with PDI-poor plasma (Fig.5B–C). However, most cells remained viable after 24h with PDI-poor or PDI-rich plasmas, similarly to FBS-incubated controls (Suppl.Fig.S5-A). Thus, EC incubation with PDI-poor or PDI-rich plasma recapitulated a gene expression pattern reminiscent of corresponding plasma protein signatures.

To further pursue this connection, we analyzed the EC secretome profile in preparations run in parallel with those used for gene expression studies. After 24h-incubation with PDI-poor or PDI-rich plasma, EC cultures were carefully washed from plasma-supplemented medium and incubated for an additional 8h-period with serum-free media, after which the conditioned medium was collected and submitted to shotgun mass spectrometry-based proteomic analysis. We identified 873

proteins across all conditions: comparison between proteins upregulated with PDI-poor or PDI-rich plasma vs. FBS controls allowed identification of 26 and 60 proteins, respectively (Suppl.Tables S4–5). Consistent with Fig.3 results in whole plasma, EC secretome showed differential protein expression with PDI-poor vs. PDI-rich plasma (Fig.6), although differences were more subtle. Coagulation-related proteins, for example, shared some degree of protein identities between PDI-poor and PDI-rich plasma, such as fibrinogen chains and histidine-rich glycoprotein. Also, both conditions associated with proteins involved in immuno-inflammation and antigen-dependent immune response. However, more detailed analysis of proteins involved in these and other pathways/processes uncovered relevant differences in gene ontology profile (Suppl.Fig.S4-C). With PDI-rich plasma, EC secretome preferentially associated with protein folding/unfolded protein response (e.g., BiP) and transport pathways (e.g. transthyretin, serotransferrin), while PDI-poor plasma preferentially associated with proteolysis processes and immune regulation (Fig.6, Suppl.Fig.S4-D). Of note, PDI, ERp57 (PDIA3) and ERp72 (PDIA4), were preferentially secreted following incubation with PDI-rich but not PDI-poor plasma vs. FBS (Suppl.Fig.S5-B and Suppl.Table S5). Importantly, there was no evidence of cell lysis, since β -actin, a validated EC damage indicator in this assay¹⁰, was undetectable in conditioned medium (Suppl.Fig.S5-B).

Functional correlates of distinct PDI-associated protein signatures in EC

Having shown correlation of specific protein signatures and associated EC phenotypes with plasma PDI levels, we inquired whether the distinct observed plasma profiles correlate with functional EC outcomes. For that, we first assessed EC adhesion. Incubation with PDI-poor plasma impaired EC attachment to fibronectin by *ca.*30% vs. FBS-control ($p < 0.01$) and was *ca.*20% lower than with PDI-rich plasma ($p < 0.05$), while the later condition was not different vs. FBS-control (Fig.7A). In fact, total plasma proteomics (Fig.3) showed many adhesion/cell matrix pathways to be preferentially related to PDI-rich plasma. Analogous experiments with EC attachment to collagen-1 showed no difference among exposures to FBS or plasmas (Suppl.Fig.S5-C).

We also investigated effects of plasma exposure in EC migration. EC monolayers incubated as described above with FBS, PDI-poor or PDI-rich plasma for 24h underwent wound-healing assays. FBS-exposed EC showed the highest migration rates (Fig.7B–C) and completely covered the wounded area after 24h. In contrast, wound repair was impaired vs. FBS-control to a greater extent following incubation with PDI-poor plasma than with PDI-rich plasma (to 53% and 72% wound area coverage, respectively, $p < 0.001$) (Fig.7C). Interestingly, both PDI-poor and PDI-rich plasma yielded analogous migration rates up to *ca.*16h of incubation, coincident with 63–65% wound recovery. However, after this stage, EC incubated with PDI-rich plasma continued to migrate to reach 72% covered area at 24h, while EC incubated with PDI-poor plasma continued to migrate, but mainly in the reverse direction, again re-expanding the wounded area, which had 53% coverage after 24h (Fig.7B–C, Suppl.Videos S1–4). Incubation of starved EC with recombinant PDI concentrations up to levels close to those of PDI-rich plasma did not enhance cell migration (not shown). Overall, these results indicate contrasting functional effects of PDI-rich vs. PDI-poor plasma in EC.

DISCUSSION

Our results indicate that plasma PDI is detectable in healthy individuals, depicting high inter-individual but low intra-individual variability. Remarkably, PDI levels behave as reporters of distinct plasma protein signatures, with PDI-rich plasma differentially expressing proteins related to cell differentiation, protein processing and housekeeping functions, among others, while PDI-poor plasma differentially displays a subset of proteins associated with coagulation, inflammatory responses and immunoactivation. Moreover, there is close correlation of PDI levels and corresponding protein signatures with EC function and phenotype, since cultured EC incubated with PDI-poor plasma recapitulated gene expression and secretome profiles in line with the observed plasma proteome. Furthermore, such signatures directly translated into functional responses, with PDI-poor plasma promoting impaired EC adhesion to fibronectin and disturbed pattern of organized migration vs. PDI-rich plasma. Essex et al²⁸ also showed a detectable plasma PDI pool in healthy donors measured by noncommercial ELISA, however at a 250–1000 ng/mL range (~4–17 nM), significantly higher than in our assays. Reasons for such distinct levels may likely relate to assay conditions and population characteristics. Also, PDI and ERp57 were previously reported in plasma proteome but not clearly associated with cardiovascular risk⁵⁴. While this is to our knowledge the first report assessing plasma concentrations of a PDI family protein, it is not unlikely that other PDIs can also potentially be present in plasma and display analogous behaviors.

While our results indicate that plasma PDI pool is mainly reduced (Fig.2D), correlations between PDI redox state and activity deserve further discussion. Oxidized PDI can directly introduce disulfides into its substrates, with reduced PDI as end-product; in the ER PDI is reoxidized by Ero1, GSSG and oxidized Prx4 or Gpx7/8³. Reduced PDI can either reduce substrate thiols (with oxidized PDI as end-product) or promote thiol reshuffling of disulfide bonds, yielding reduced PDI^{7, 55}. Interestingly, kinetic trapping experiments suggest that different substrates are biased to reduction vs. oxidation by PDI. The first comprises platelet substrates including, among others, platelet factor-V²⁴, annexin V, ERp57²³ and plasma substrates such as vitronectin, some complement factors, HRG and thrombospondin-1²². In contrast, cathepsin G, glutaredoxin-1, thioredoxin, GP1b and fibrinogen are biased for PDI-dependent oxidation in platelets²³. In parallel, although PDI does not seem a mass peroxide sensor as discussed before, it is affected by redox potentials⁷. Accordingly, one might expect that plasma PDI would be primarily oxidized⁵⁶, contrary to our results, since redox potential of the major plasma thiol pools, Cys/CySS (−80±9 mV) and GSH/GSSG (−137±9 mV)¹ are more oxidizing than PDI redox potential (−190±10 mV). While mechanisms underlying this apparent paradox are yet unclear, this is not unexpected since most plasma thiol pools are not in equilibrium¹, suggesting possible mechanism(s) for their mutual insulation.

Several mechanisms may regulate plasma PDI levels and account for their variability. Our data (Suppl.Fig.5-B, Suppl.Table S5) showed increased PDI secretion by cultured EC exposed to PDI-rich plasma, thus suggesting that specific plasma factors control PDI externalization from EC. Contrarily, platelet activity was similar in individuals with low vs. high plasma PDI, suggesting that PDI level variability is not explained only by distinct amounts of secretion from platelets, which has been well-documented during thrombus formation³⁰. In contrast, increased platelet PDI secretion was reported in patients with hemophilia A⁵⁷ and a pH-dependent release from platelets has been

suggested as possible source of plasma PDI²⁸. Meanwhile, there was decrease in soluble PDI following platelet activation (Fig.4E), which we attribute to sequestration of platelet-derived PDI by its potential substrates^{22, 23, 58}. In fact, PDI is retained in platelets through binding to surface $\beta 3$ integrins^{56, 59}, and several PDI substrates have been identified in platelet-rich plasma²¹. A similar effect likely occurred with respect to other circulating or EC-attached PDI substrates, which may account for its plasma level variability. Finally, recent observations suggest that single nucleotide polymorphisms are a substantial source of plasma protein variability⁶⁰, an issue deserving further investigation for PDI. Overall levels of *pec*PDI represent a minor fraction of total intracellular PDI pool, but can increase following forced PDI overexpression⁶¹. Moreover, it is important to point that our study addressed healthy individuals, while disease processes associated with ongoing vascular injury and inflammation may enhance circulating PDI levels due to cell death/leakage²¹.

An important result was the contrasting functional effects of PDI-rich plasma, which supported organized EC migration, vs. PDI-poor plasma, which significantly disrupted the migration pattern. In both cases, such effects are likely related to corresponding plasma protein signatures rather than to PDI levels themselves. This is because the pM/nM PDI concentration ranges contrast with higher concentrations of its many plasma substrates (e.g., HRG, 100-150 $\mu\text{g}/\text{mL}$)⁴⁵, other thiol proteins (400–600 μM) and low-molecular weight thiols (7.3–9.0 μM for glutathione and 202–281 μM for cysteine¹). Thus, it is likely that PDI levels behave as a reporter of the amount and type of bound substrates.

PDI adds to other thiol-proteins with potential as disease biomarkers such as glutathione peroxidase-3, quiescin sulfhydryl oxidase, thioredoxin/thioredoxin reductase, glutaredoxin¹ and S-nitrosogluthathione reductase⁶². However, except for a described association of glutaredoxin-1 with endothelial permeability⁶³, functional endothelial effects related to such proteins remain unknown, as well as their possible correlation with plasma protein signatures. In fact, to the best of our knowledge, our study is the first to describe different plasma proteome profiles and corresponding EC phenotypes related to vascular (dys)function and thus our data open new ways to understand how plasma biomarkers in general, and specifically redox biomarkers, can interplay with disease processes. A clear limitation of our results is that the type of population and the small number of subjects does not allow inferences about biomarker capabilities of PDI levels. Nevertheless, to get a potential insight into this question, we evaluated PDI levels from plasma samples ($n=95$) collected in a previously well-described and validated databank from a population composed of individuals with clinically evident vascular disease⁶⁴. Patients who had documented cardiovascular events, namely myocardial infarction, unstable angina and cerebrovascular accident (further detailed at Table 2) showed significantly lower median circulating PDI levels vs. healthy individuals (Fig.7D), indicating that lower PDI levels may have a general correlation with enhanced cardiovascular risk in patients with chronic atherosclerotic disease. Again, PDI levels were unrelated to other traditional risk factors (gender, LDL-cholesterol levels, HDL-cholesterol levels, triglycerides, diabetes, body mass index – Suppl. Table S6) except age (which showed an association, $p<0.05$). These data are in line with our proteomic and functional studies in healthy individuals, but, again, further studies are needed to assess general applicability of PDI levels as risk biomarkers.

Overall, our results reinforce that plasma proteins including PDI are both contributors and accessible reporters of disease-associated conditions⁶⁵ and provide evidence that these concepts also extend to healthy individuals. Improved identification of suitable reporters, particularly those

related to PDI, can yield insights into disease mechanisms involving endothelium-related plasma protein signatures. This redox-related protein may pave the way the innovative concept of endothelial-related liquid biopsy.

FUNDING

This work was supported by the CEPID Redoxoma- FAPESP (Fundação de Amparo à Pesquisa do Estado de Sao Paulo) grant 2013/07937-8, in addition to scholarship grants 2014/20595-1 (to PVSO), 2017/19866-9 (to NTS) and 2018/07511-4 (to TCDB). FAPESP grant 2013/25177-0, Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq–Grant 305245/2015-5 and Fundação Butantan (to MLS). CAPES (Coordenação de Aperfeiçoamento de Pessoa de Nível Superior) fellowship (to ATAS). FAPESP grants 2013/08711-3, 2014/10068-4 and 2017/25588-1 (to DM-S). CAPES-PROEX grant 154529 (to SG-R).

DECLARATION OF INTEREST

None.

ACKNOWLEDGEMENTS

We thank Laura Ventura and Carolina Fernandes for technical support with blood collection procedure.

REFERENCES

1. Oliveira PVS, Laurindo FRM. Implications of plasma thiol redox in disease. *Clin Sci (Lond)*. 2018;132:1257-1280
2. Ellgaard L, Ruddock LW. The human protein disulphide isomerase family: Substrate interactions and functional properties. *EMBO Rep*. 2005;6:28-32
3. Laurindo FR, Pescatore LA, Fernandes DeC. Protein disulfide isomerase in redox cell signaling and homeostasis. *Free Radic Biol Med*. 2012;52:1954-1969
4. Appenzeller-Herzog C, Ellgaard L. The human pdi family: Versatility packed into a single fold. *Biochim Biophys Acta*. 2008;1783:535-548
5. Wang L, Wang X, Wang CC. Protein disulfide-isomerase, a folding catalyst and a redox-regulated chaperone. *Free Radic Biol Med*. 2015;83:305-313
6. Tanaka LY, Araújo HA, Hironaka GK, Araujo TL, Takimura CK, Rodriguez AI, Casagrande AS, Gutierrez PS, Lemos-Neto PA, Laurindo FR. Peri/epicellular protein disulfide isomerase sustains vascular lumen caliber through an anticonstrictive remodeling effect. *Hypertension*. 2016;67:613-622
7. Soares Moretti AI, Martins Laurindo FR. Protein disulfide isomerases: Redox connections in and out of the endoplasmic reticulum. *Arch Biochem Biophys*. 2017;617:106-119
8. Bekendam RH, Flaumenhaft R. Inhibition of protein disulfide isomerase in thrombosis. *Basic Clin Pharmacol Toxicol*. 2016;119 Suppl 3:42-48

9. Zhou J, Wu Y, Wang L, Rauova L, Hayes VM, Poncz M, Essex DW. The c-terminal cghc motif of protein disulfide isomerase supports thrombosis. *J Clin Invest.* 2015;125:4391-4406
10. Araujo TLS, Zeidler JD, Oliveira PVS, Dias MH, Armelin HA, Laurindo FRM. Protein disulfide isomerase externalization in endothelial cells follows classical and unconventional routes. *Free Radic Biol Med.* 2017;103:199-208
11. Jasuja R, Passam FH, Kennedy DR, Kim SH, van Hessem L, Lin L, Bowley SR, Joshi SS, Dilks JR, Furie B, Furie BC, Flaumenhaft R. Protein disulfide isomerase inhibitors constitute a new class of antithrombotic agents. *J Clin Invest.* 2012;122:2104-2113
12. Sharda A, Kim SH, Jasuja R, Gopal S, Flaumenhaft R, Furie BC, Furie B. Defective pdi release from platelets and endothelial cells impairs thrombus formation in hermansky-pudlak syndrome. *Blood.* 2015;125:1633-1642
13. Crescente M, Pluthero FG, Li L, Lo RW, Walsh TG, Schenk MP, Holbrook LM, Louriero S, Ali MS, Vaiyapuri S, Falet H, Jones IM, Poole AW, Kahr WH, Gibbins JM. Intracellular trafficking, localization, and mobilization of platelet-borne thiol isomerases. *Arterioscler Thromb Vasc Biol.* 2016;36:1164-1173
14. Bekendam RH, Bendapudi PK, Lin L, Nag PP, Pu J, Kennedy DR, Feldenzer A, Chiu J, Cook KM, Furie B, Huang M, Hogg PJ, Flaumenhaft R. A substrate-driven allosteric switch that enhances pdi catalytic activity. *Nat Commun.* 2016;7:12579
15. Xu S, Butkevich AN, Yamada R, Zhou Y, Debnath B, Duncan R, Zandi E, Petasis NA, Neamati N. Discovery of an orally active small-molecule irreversible inhibitor of protein disulfide isomerase for ovarian cancer treatment. *Proc Natl Acad Sci U S A.* 2012;109:16348-16353
16. Xu S, Sankar S, Neamati N. Protein disulfide isomerase: A promising target for cancer therapy. *Drug Discov Today.* 2014;19:222-240
17. Stopa JD, Zwicker JI. The intersection of protein disulfide isomerase and cancer associated thrombosis. *Thromb Res.* 2018;164 Suppl 1:S130-S135
18. Essex DW, Wu Y. Multiple protein disulfide isomerases support thrombosis. *Curr Opin Hematol.* 2018;25:395-402
19. Essex DW. Redox control of platelet function. *Antioxid Redox Signal.* 2009;11:1191-1225
20. Mor-Cohen R. Disulfide bonds as regulators of integrin function in thrombosis and hemostasis. *Antioxid Redox Signal.* 2016;24:16-31
21. Sharda A, Furie B. Regulatory role of thiol isomerases in thrombus formation. *Expert Rev Hematol.* 2018;11:437-448
22. Bowley SR, Fang C, Merrill-Skoloff G, Furie BC, Furie B. Protein disulfide isomerase secretion following vascular injury initiates a regulatory pathway for thrombus formation. *Nat Commun.* 2017;8:14151
23. Stopa JD, Baker KM, Grover SP, Flaumenhaft R, Furie B. Kinetic-based trapping by intervening sequence variants of the active sites of protein-disulfide isomerase identifies platelet protein substrates. *J Biol Chem.* 2017;292:9063-9074
24. Stopa JD, Neuberger D, Puligandla M, Furie B, Flaumenhaft R, Zwicker JI. Protein disulfide isomerase inhibition blocks thrombin generation in humans by interfering with platelet factor v activation. *JCI Insight.* 2017;2:e89373
25. Peixoto Á, Geyer RR, Iqbal A, Truzzi DR, Soares Moretti AI, Laurindo FRM, Augusto O. Peroxynitrite preferentially oxidizes the dithiol redox motifs of protein-disulfide isomerase. *J Biol Chem.* 2018;293:1450-1465
26. Kim J, Mayfield SP. Protein disulfide isomerase as a regulator of chloroplast translational activation. *Science.* 1997;278:1954-1957
27. Fernandes DC, Manoel AH, Wosniak J, Laurindo FR. Protein disulfide isomerase overexpression in vascular smooth muscle cells induces spontaneous preemptive nadph

- oxidase activation and nox1 mRNA expression: Effects of nitrosothiol exposure. *Arch Biochem Biophys*. 2009;484:197-204
28. Essex DW, Miller A, Swiatkowska M, Feinman RD. Protein disulfide isomerase catalyzes the formation of disulfide-linked complexes of vitronectin with thrombin-antithrombin. *Biochemistry*. 1999;38:10398-10405
 29. Chen K, Lin Y, Detwiler TC. Protein disulfide isomerase activity is released by activated platelets. *Blood*. 1992;79:2226-2228
 30. Chen K, Detwiler TC, Essex DW. Characterization of protein disulphide isomerase released from activated platelets. *Br J Haematol*. 1995;90:425-431
 31. Banfi C, Brioschi M, Wait R, Begum S, Gianazza E, Pirillo A, Mussoni L, Tremoli E. Proteome of endothelial cell-derived procoagulant microparticles. *Proteomics*. 2005;5:4443-4455
 32. Raturi A, Miersch S, Hudson JW, Mutus B. Platelet microparticle-associated protein disulfide isomerase promotes platelet aggregation and inactivates insulin. *Biochim Biophys Acta*. 2008;1778:2790-2796
 33. Fan GQ, Qin RR, Li YH, Song DJ, Chen TS, Zhang W, Zhong M, Zhang Y, Xing YQ, Wang ZH. Endothelial cells microparticle-associated protein disulfide isomerase promotes platelet activation in metabolic syndrome. *Oncotarget*. 2016;7:83231-83240
 34. Iqbal A, Paviani V, Moretti AI, Laurindo FR, Augusto O. Oxidation, inactivation and aggregation of protein disulfide isomerase promoted by the bicarbonate-dependent peroxidase activity of human superoxide dismutase. *Arch Biochem Biophys*. 2014;557:72-81
 35. Raturi A, Mutus B. Characterization of redox state and reductase activity of protein disulfide isomerase under different redox environments using a sensitive fluorescent assay. *Free Radic Biol Med*. 2007;43:62-70
 36. Montano SJ, Lu J, Gustafsson TN, Holmgren A. Activity assays of mammalian thioredoxin and thioredoxin reductase: Fluorescent disulfide substrates, mechanisms, and use with tissue samples. *Anal Biochem*. 2014;449:139-146
 37. They C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*. 2006;Chapter 3:Unit 3 22
 38. Giannakopoulos B, Gao L, Qi M, Wong JW, Yu DM, Vlachoyiannopoulos PG, Moutsopoulos HM, Atsumi T, Koike T, Hogg P, Qi JC, Krilis SA. Factor xi is a substrate for oxidoreductases: Enhanced activation of reduced fxi and its role in antiphospholipid syndrome thrombosis. *J Autoimmun*. 2012;39:121-129
 39. Moretti AIS, Pavanelli JC, Nolasco P, Leisegang MS, Tanaka LY, Fernandes CG, Wosniak J, Jr., Kajihara D, Dias MH, Fernandes DC, Jo H, Tran NV, Ebersberger I, Brandes RP, Bonatto D, Laurindo FRM. Conserved gene microsynteny unveils functional interaction between protein disulfide isomerase and rho guanine-dissociation inhibitor families. *Sci Rep*. 2017;7:17262
 40. Moraes MS, Costa PE, Batista WL, Paschoalin T, Curcio MF, Borges RE, Taha MO, Fonseca FV, Stern A, Monteiro HP. Endothelium-derived nitric oxide (no) activates the no-epidermal growth factor receptor-mediated signaling pathway in bradykinin-stimulated angiogenesis. *Arch Biochem Biophys*. 2014;558:14-27
 41. Garcia S, Silva-Costa LC, Reis-de-Oliveira G, Guest PC, Baldasso PA, Cassoli JS, Martins-de-Souza D. Identifying biomarker candidates in the blood plasma or serum proteome. *Adv Exp Med Biol*. 2017;974:193-203
 42. Tang S, Morgan KG, Parker C, Ware JA. Requirement for protein kinase c theta for cell cycle progression and formation of actin stress fibers and filopodia in vascular endothelial cells. *J Biol Chem*. 1997;272:28704-28711

43. Lin L, Gopal S, Sharda A, Passam F, Bowley SR, Stopa J, Xue G, Yuan C, Furie BC, Flaumenhaft R, Huang M, Furie B. Quercetin-3-rutinoside inhibits protein disulfide isomerase by binding to its b'x domain. *J Biol Chem*. 2015;290:23543-23552
44. Prado GN, Romero JR, Rivera A. Endothelin-1 receptor antagonists regulate cell surface-associated protein disulfide isomerase in sickle cell disease. *FASEB J*. 2013;27:4619-4629
45. Jones AL, Hulett MD, Parish CR. Histidine-rich glycoprotein: A novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems. *Immunol Cell Biol*. 2005;83:106-118
46. Tsuchida-Straeten N, Ensslen S, Schäfer C, Wöltje M, Denecke B, Moser M, Gräber S, Wakabayashi S, Koide T, Jahnen-Dechent W. Enhanced blood coagulation and fibrinolysis in mice lacking histidine-rich glycoprotein (hrp). *J Thromb Haemost*. 2005;3:865-872
47. Druhan LJ, Lance A, Li S, Price AE, Emerson JT, Baxter SA, Gerber JM, Avalos BR. Leucine rich α -2 glycoprotein: A novel neutrophil granule protein and modulator of myelopoiesis. *PLoS One*. 2017;12:e0170261
48. Frey EA, Miller DS, Jahr TG, Sundan A, Bazil V, Espevik T, Finlay BB, Wright SD. Soluble cd14 participates in the response of cells to lipopolysaccharide. *J Exp Med*. 1992;176:1665-1671
49. Jasuja R, Furie B, Furie BC. Endothelium-derived but not platelet-derived protein disulfide isomerase is required for thrombus formation in vivo. *Blood*. 2010;116:4665-4674
50. Sumpio BE, Riley JT, Dardik A. Cells in focus: Endothelial cell. *Int J Biochem Cell Biol*. 2002;34:1508-1512
51. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol*. 2007;7:803-815
52. Badrichani AZ, Stroka DM, Bilbao G, Curiel DT, Bach FH, Ferran C. Bcl-2 and bcl-xl serve an anti-inflammatory function in endothelial cells through inhibition of nf-kappab. *J Clin Invest*. 1999;103:543-553
53. Damgaard RB, Gyrd-Hansen M. Inhibitor of apoptosis (iap) proteins in regulation of inflammation and innate immunity. *Discov Med*. 2011;11:221-231
54. Ganz P, Heidecker B, Hveem K, Jonasson C, Kato S, Segal MR, Sterling DG, Williams SA. Development and validation of a protein-based risk score for cardiovascular outcomes among patients with stable coronary heart disease. *JAMA*. 2016;315:2532-2541
55. Hudson DA, Gannon SA, Thorpe C. Oxidative protein folding: From thiol-disulfide exchange reactions to the redox poise of the endoplasmic reticulum. *Free Radic Biol Med*. 2015;80:171-182
56. Flaumenhaft R, Furie B. Vascular thiol isomerases. *Blood*. 2016;128:893-901
57. Voigtlaender M, Holstein K, Spath B, Bokemeyer C, Langer F. Expression and release of platelet protein disulphide isomerase in patients with haemophilia a. *Haemophilia*. 2016;22:e537-e544
58. Flaumenhaft R. Advances in vascular thiol isomerase function. *Curr Opin Hematol*. 2017;24:439-445
59. Cho J, Kennedy DR, Lin L, Huang M, Merrill-Skoloff G, Furie BC, Furie B. Protein disulfide isomerase capture during thrombus formation in vivo depends on the presence of β 3 integrins. *Blood*. 2012;120:647-655
60. Joshi A, Mayr M. In aptamers they trust. *Circulation*. 2018;138:2482-2485
61. Araujo TLS, Fernandes CG, Laurindo FRM. Golgi-independent routes support protein disulfide isomerase externalization in vascular smooth muscle cells. *Redox Biol*. 2017;12:1004-1010
62. Hayashida K, Bagchi A, Miyazaki Y, Hirai S, Seth D, Silverman MG, Rezoagli E, Marutani E, Mori N, Magliocca A, Liu X, Berra L, Hindle AG, Donnino MW, Malhotra R,

- Bradley MO, Stamler JS, Ichinose F. Improvement in outcomes after cardiac arrest and resuscitation by inhibition of s-nitrosoglutathione reductase. *Circulation*. 2018
63. Han J, Weisbrod RM, Shao D, Watanabe Y, Yin X, Bachschmid MM, Seta F, Janssen-Heininger YMW, Matsui R, Zang M, Hamburg NM, Cohen RA. The redox mechanism for vascular barrier dysfunction associated with metabolic disorders: Glutathionylation of rac1 in endothelial cells. *Redox Biol*. 2016;9:306-319
64. Jannes CE, Santos RD, de Souza Silva PR, Turolla L, Gagliardi AC, Marsiglia JD, Chacra AP, Miname MH, Rocha VZ, Filho WS, Krieger JE, Pereira AC. Familial hypercholesterolemia in brazil: Cascade screening program, clinical and genetic aspects. *Atherosclerosis*. 2015;238:101-107
65. Vegvari A, Welinder C, Lindberg H, Fehniger TE, Marko-Varga G. Biobank resources for future patient care: Developments, principles and concepts. *J Clin Bioinforma*. 2011;1:24

FIGURES AND FIGURE LEGENDS

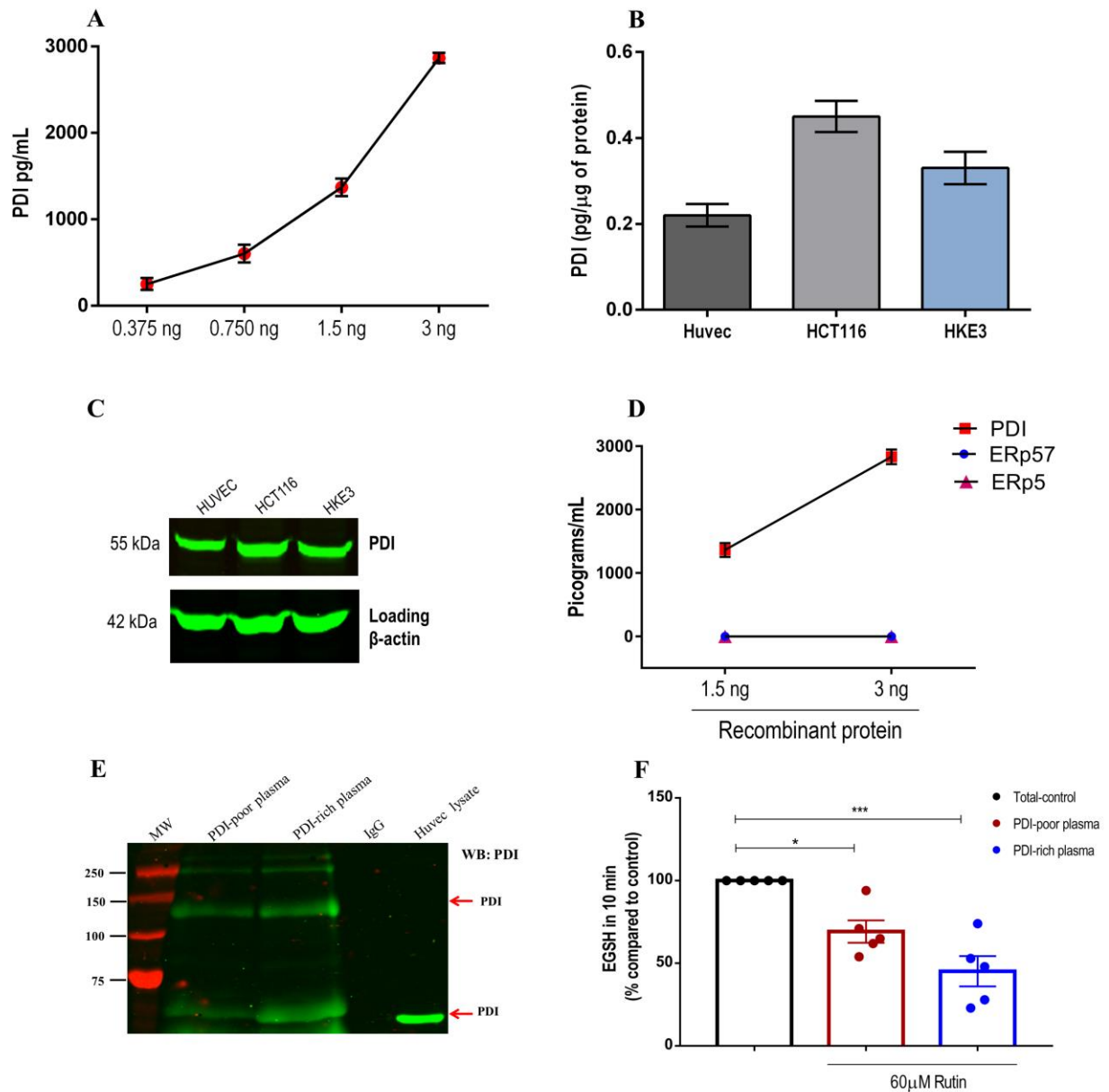


Figure 1. Validation of ELISA assay for specific detection of PDI. **A.** PDI concentration curves (0.375 to 3 ng/mL) were generated using purified PDI quantified by ELISA. Measurements were analyzed on 4 independent assays. Data represent mean±SEM. **B-C.** Human cell lysates (HUVEC, HCT116 and HKE3) were (B) quantified by ELISA ($n=4$; mean±SEM) and (C) submitted to protein separation by reducing SDS-PAGE and immunoblotted with anti-PDI ELISA's capture antibody or anti-β actin (loading control) ($n=3$). Uncropped western blots are shown in Supplementary Fig.S6. **D.** Cross-reactivity with other PDI family members was analyzed using recombinant PDI, ERp57 and ERp5 (1.5–3 ng/mL) quantified by ELISA. Measurements were analyzed on 4 independent

assays. Data represent mean \pm SEM. **E.** Plasma PDI immunoprecipitation. Platelet-poor plasma was diluted in lysis buffer and immunoprecipitated using rabbit anti-PDI antibody. Immunoblotting was performed using mouse anti-PDI (RL90) ($n=3$ from independent experiments). **F.** Detection of plasma PDI reductase activity (di-eosin-GSSG assay) by formation of the reduced fluorogenic product eosin-5-isothiocyanate-coupled reduced glutathione (EGSH). It was measured in platelet-poor plasma exposed or not to 60 μ M of rutin. Bar graphs represent the percentage cleavage of di-eosin-GSSG as compared to total reductase activity – Control (100%) in 10 min. The main result in this case is the rutin-inhibitable fraction of di-eosin-GSSG reductase activity. Data represent mean \pm SEM from 5 independent experiments. * $p<0.05$; *** $p<0.001$ vs. control (One-way ANOVA followed by Tukey's post test).

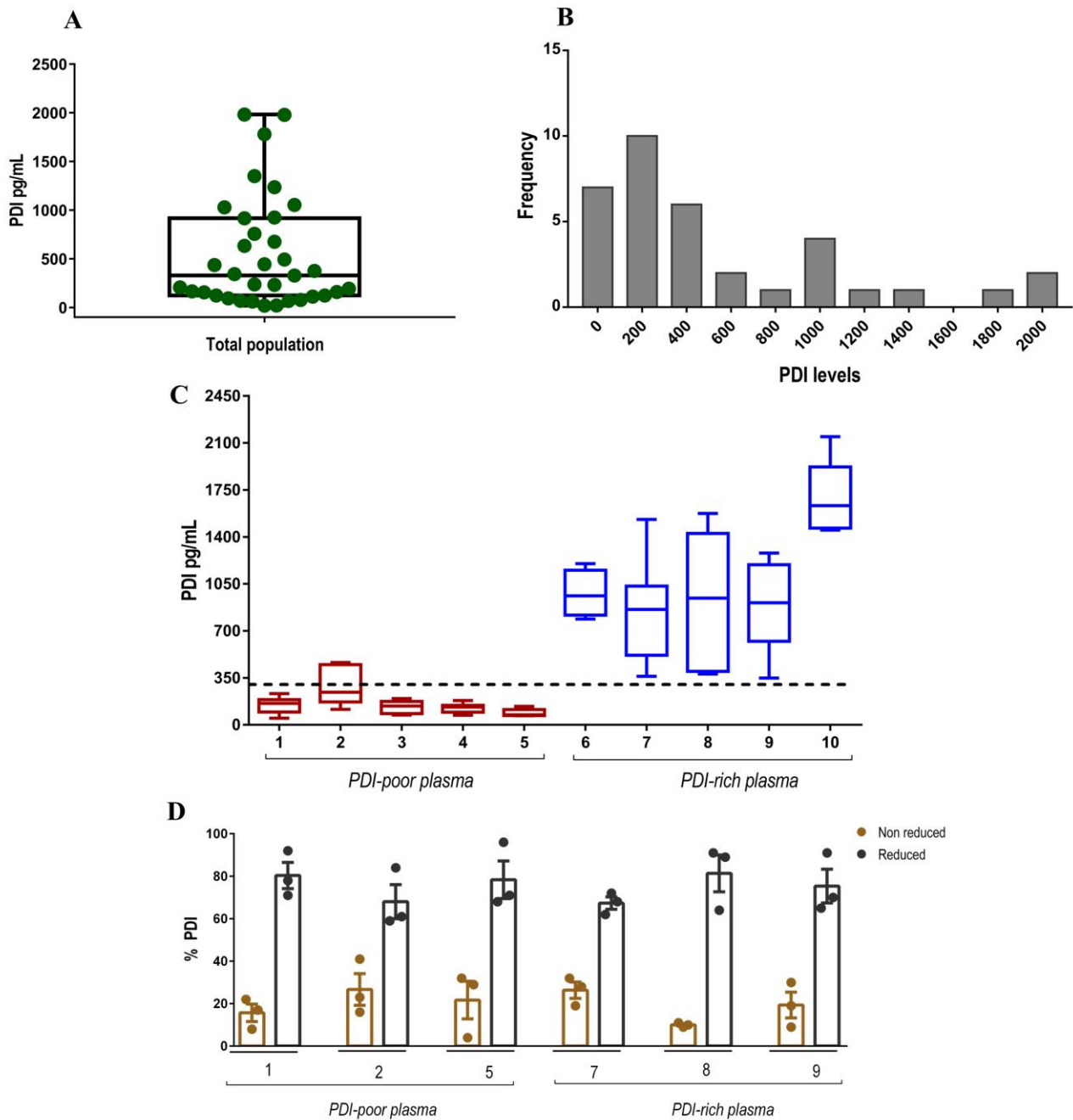


Figure 2. Plasma PDI levels in healthy individuals. **A.** Distribution of plasma PDI levels are represented by box plot, indicating the median plus lower and upper quartiles ($n=35$). **B.** Frequency distribution of plasma PDI levels shown in (A). **C.** Graphic representation of plasma PDI from selected individuals assessed over time. Each box plot represents one individual and their different plasma samples collected over time ($n=10-15$ samples for each individual), as detailed in the text. The dashed line designates the median value (330 pg/mL) of the whole population shown in (A). Values ≤ 330 pg/mL were designated as PDI-poor plasma and >330 pg/mL as PDI-rich plasma. Data represent median plus lower and upper quartiles. **D.** Detection of PDI redox state in human plasma. Plasma samples labelled with either EZ-Link Sulfo-NHS-Biotin or MPB were acetone precipitated (to remove unbounded biotinylated probes) and protein pellets were re-suspended in PBS-tween. Samples were added to a plate coated with anti-PDI antibody and after this incubation probed with a monoclonal antibody to biotin (peroxidase conjugate). Bar graphics represent the percentage of reduced or non-reduced PDI comparing MPB vs. EZ-Link Sulpho-NHS-Biotin labelling. Numbers represent different individuals. Data represent mean \pm SEM from 3 independent experiments.

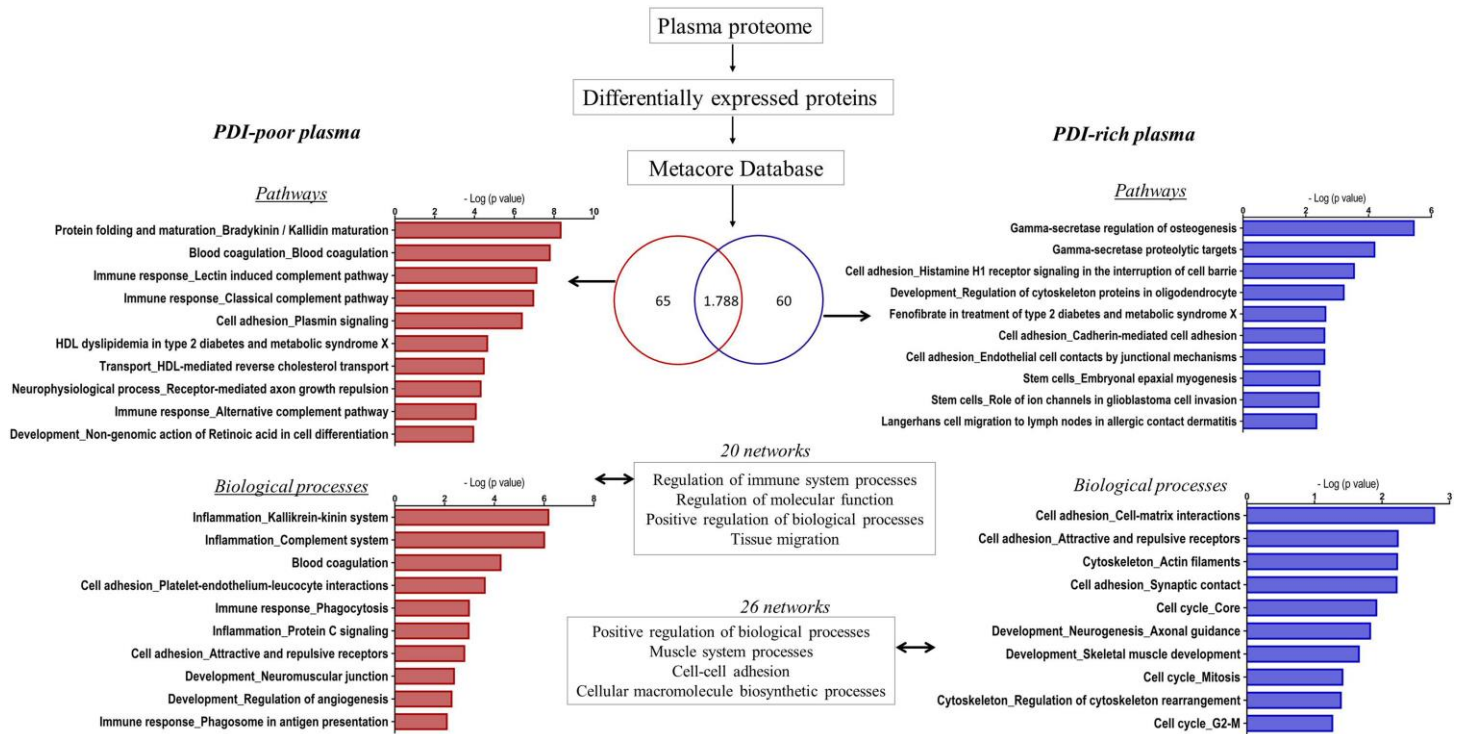


Figure 3. Total proteome and Metacore analysis of PDI-poor or PDI-rich plasma. Samples from individuals with PDI-poor plasma ($n=3$) or PDI-rich plasma ($n=3$) were submitted to shotgun mass spectrometry profiling analysis to identify proteins differentially expressed in each case. There were 65 proteins differentially upregulated in PDI-poor plasma samples and 60 proteins differentially upregulated in PDI-rich plasma samples. Further analysis using Metacore software revealed enrichment in several pathways and biological processes ($p<0.05$).

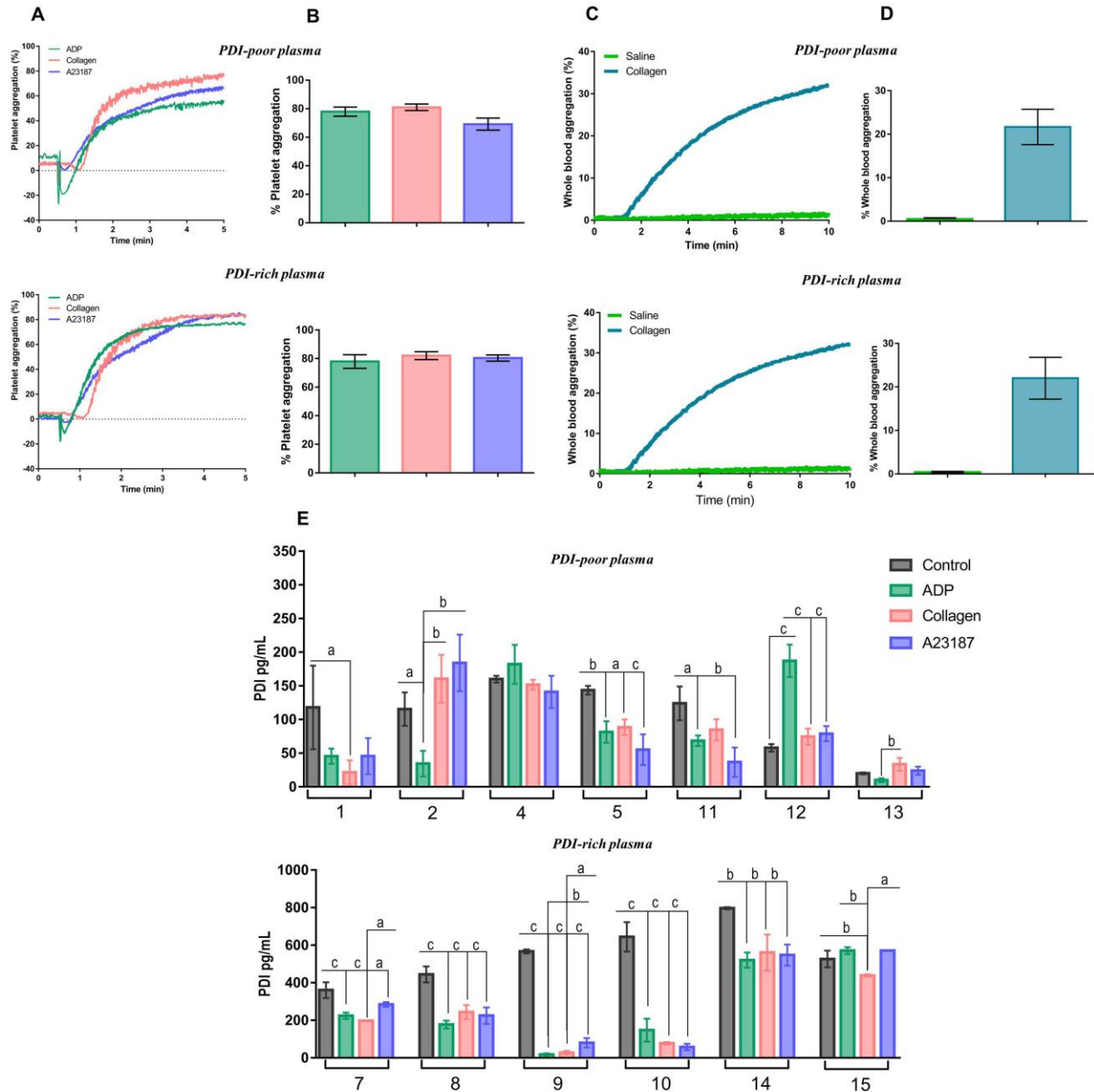


Figure 4. Platelet aggregation in individuals with PDI-poor or PDI-rich plasma. Experiments were performed in platelet-rich plasma (PRP) by light transmission and whole blood by impedance (in both cases $2\text{--}3 \times 10^8$ platelets mL^{-1} , 37°C). Platelets were stimulated with agonists and tracings were recorded for 5 min for PRP and 10 min for whole blood. **A.** Representative platelet aggregation tracings induced by $10 \mu\text{M}$ ADP, $5 \mu\text{g/mL}$ collagen and $20.5 \mu\text{M}$ A23187. **B.** Maximal extents of aggregation from (A). No difference was detected among groups. **C.** Representative whole blood aggregation tracings induced by $25 \mu\text{M}$ collagen. **D.** Maximal extents of aggregation from (C). No difference was detected between PDI-poor and PDI-rich plasma groups for stimulated aggregation. **E.** Soluble PDI was measured by ELISA in supernatants of resting (control) or activated PRP as described in Materials and Methods. Numbers represent different individuals. All data represent mean \pm SEM from 6–7 independent experiments. ^a $p < 0.05$; ^b $p < 0.005$; ^c $p < 0.0005$ (One-way ANOVA followed by Tukey’s post test, for each individual samples).

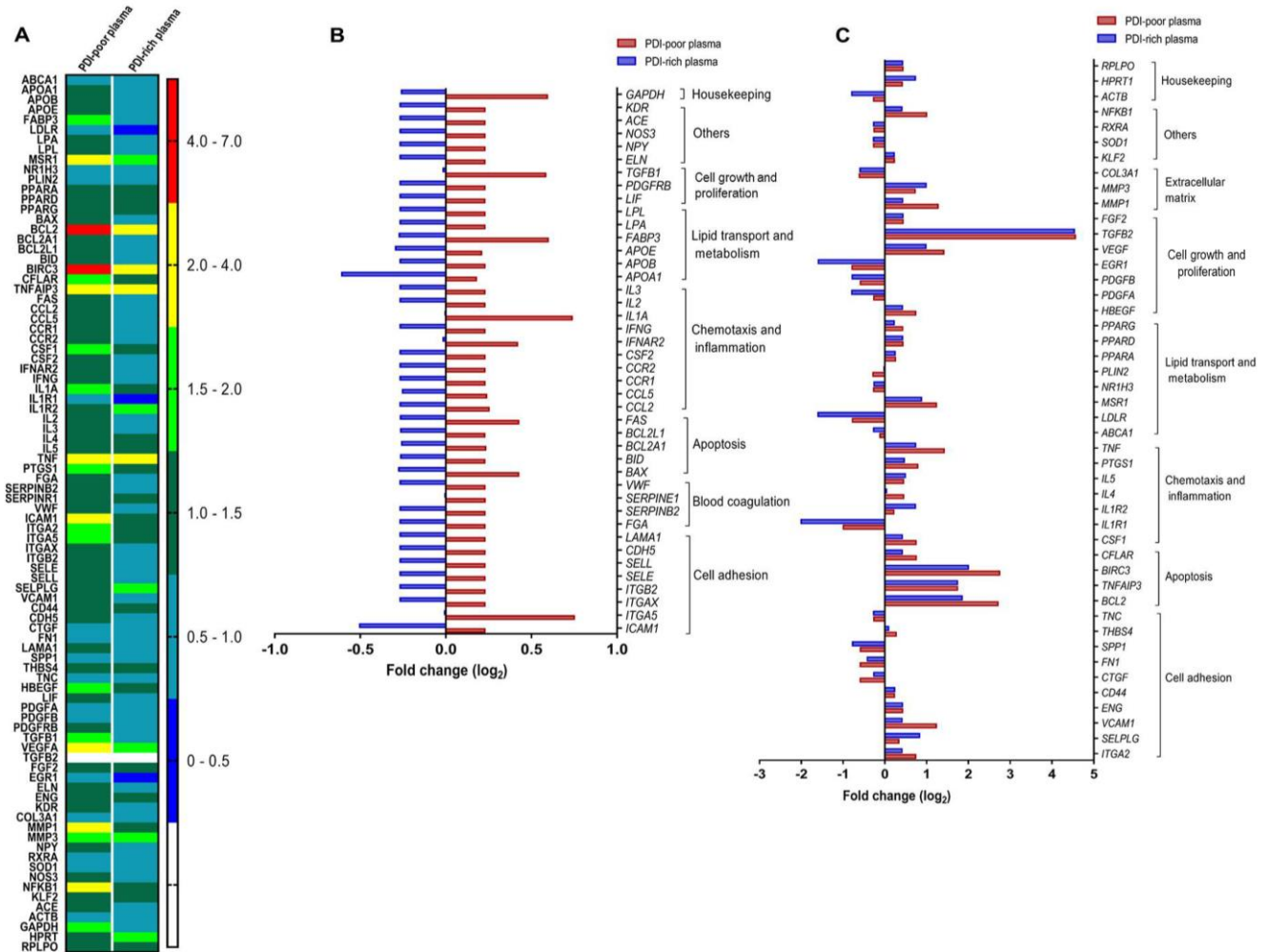


Figure 5. Effects of incubation with PDI-poor or PDI-rich plasma on endothelial cell expression of several genes covering different biological processes. EC were incubated with 5% PDI-poor plasma, PDI-rich plasma or FBS for 24h and the expression levels of pre-specified 84 genes were analyzed using RT² Profile PCR array. **A.** Heat map of relative expressions of transcripts. **B.** Transcript levels are shown as Log₂ fold-changes for genes which were simultaneously upregulated with PDI-poor and downregulated with PDI-rich plasma. **C.** Transcript levels are shown as Log₂ fold-changes for genes with distinct behavior in response to incubation with PDI-poor or PDI-rich plasma. All expression values were normalized for the expression of β -2-microglobulin. All data represent mean values from 3 independent experiments.

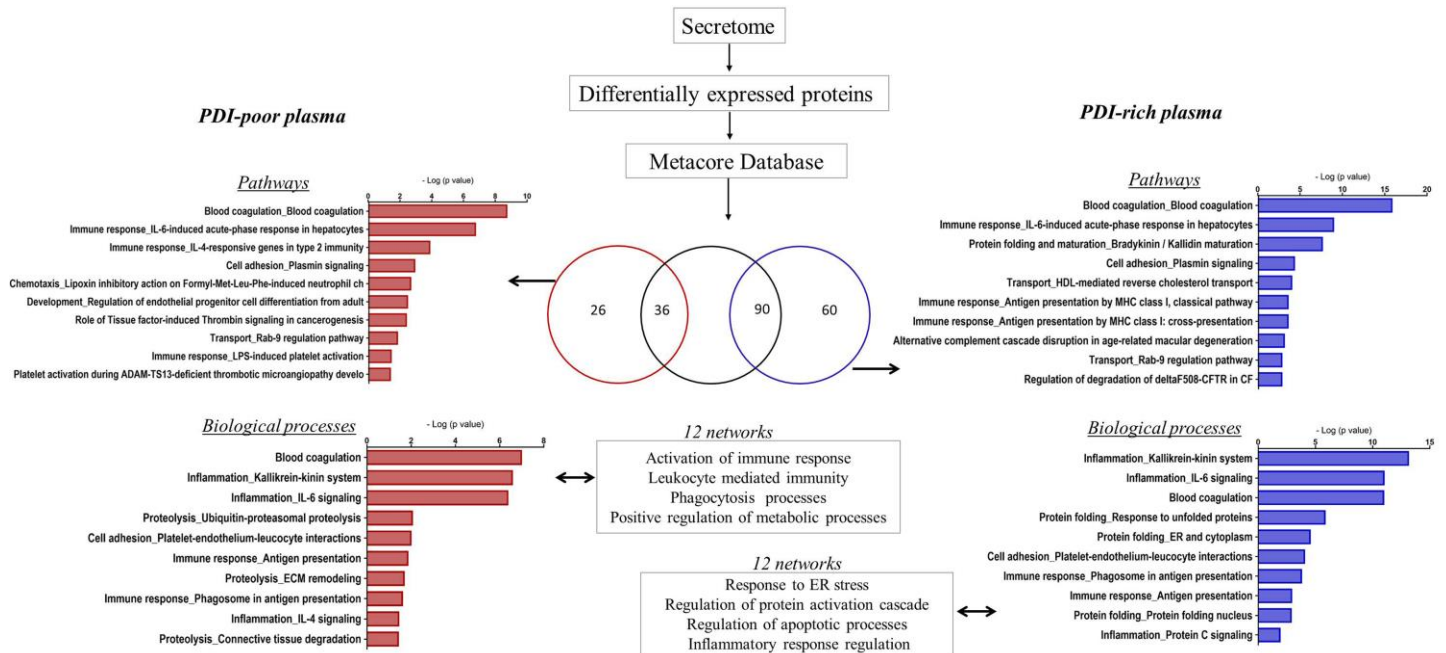


Figure 6. Secretome proteome and enrichment analysis of endothelial cells in response to incubation with PDI-poor or PDI-rich plasma. EC were incubated with 5% PDI-poor plasma, PDI-rich plasma or FBS-control for 24h and conditioned medium was generated after 8h in serum-free media for secretome analysis, as described in Materials and Methods. Proteome analysis through shotgun mass spectrometry profiling disclosed subsets of upregulated proteins differentially expressed between EC exposed to PDI-poor or PDI-rich plasma vs. FBS-control. These subsets were significantly enriched in several pathways, biological processes and gene ontology processes ($p < 0.05$), as analyzed by Metacore software. All data represent results from 3 independent experiments.

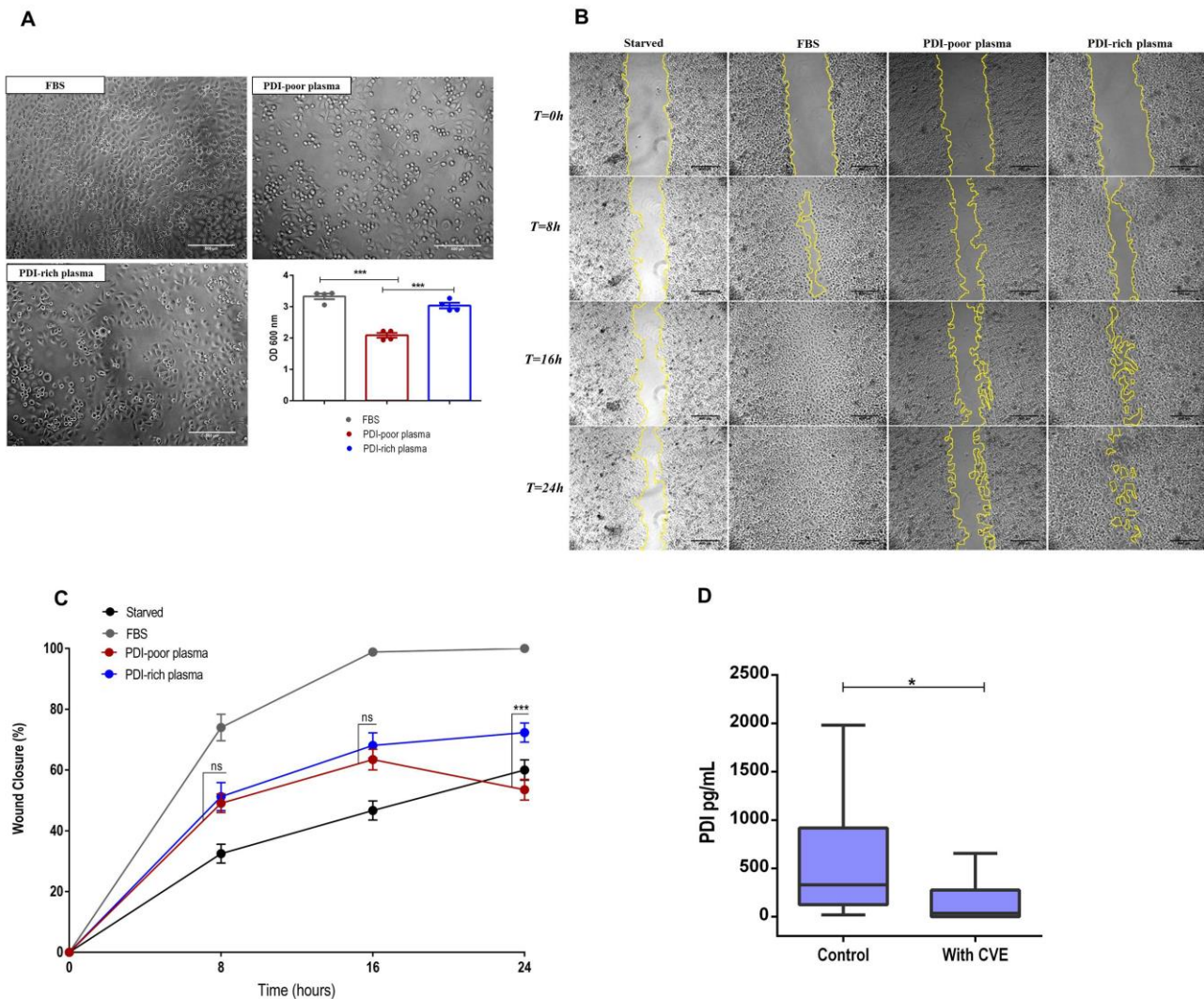


Figure 7. Functional effects of PDI-poor or PDI-rich plasma incubation in endothelial cell adhesion and migration. **A.** HUVEC were pre-incubated with 5% PDI-poor plasma, PDI-rich plasma or FBS for 24h, seeded (4×10^4 cells/mL) in 96-well plates coated with fibronectin ($5 \mu\text{g/mL}$) and allowed to adhere for 1h at 37°C . After washing, adherent cells were stained with crystal violet, solubilized by SDS and absorbance was measured at 600 nm. Scale bar: $500 \mu\text{M}$. Objective: $40\times$. Data represent mean \pm SEM from 5 independent experiments. $***p < 0.001$ (One-way ANOVA followed by Tukey's post test) **B.** HUVEC were seeded at 2×10^5 cells/mL in 24-well plates. After culturing for 48h, a wound was created by scratching with a sterile pipette tip. Cells were cultured for an additional 24h in medium as follows: without FBS (starved), with 5% of FBS-control, PDI-poor plasma or PDI-rich plasma. Photomicrographs representing migration into the denuded area at 0h, 8h, 16h and 24h. The zone between two yellow lines indicates the area occupied by the initial wound. Scale bar: $500 \mu\text{M}$. Objective: $5\times$. **C.** Percent wound surface coverage by migrating HUVEC showed in (B). Data represent mean \pm SEM from 4 independent experiments. $***p < 0.0001$ (Two-way ANOVA followed by Tukey's post test). For clarity, statistics are shown only for EC incubated with PDI-poor vs. PDI-rich plasma. **D.** Plasma PDI concentration measured by ELISA, in a population with cardiovascular events ($n=95$, see Table 4 for details) vs. healthy controls ($n=35$). Data represent median plus lower and upper quartiles. $*p < 0.05$ (Mann-Whitney t test).

TABLES

Table 1. Baseline characteristics of healthy individual population

<i>n</i>	35
Age	34.2 ± 1.6
Males (%)	40
Females (%)	60
Total cholesterol, mg/dL	186.5 ± 5.1
HDL cholesterol, mg/dL	58.3 ± 2.8
LDL cholesterol, mg/dL	111.3 ± 5.1
Non-HDL cholesterol, mg/dL	128.7 ± 5.6
Triglycerides, mg/dL	84.9 ± 8.2
Whole blood platelet count (x10 ⁹ /L)	263.6 ± 10.1
Red blood cell count (x10 ¹² /L)	4.7 ± 0.1
White blood cell count (10 ⁹ /L)	6.7 ± 0.3
C-Reactive Protein, mg/L	2.6 ± 0.8
PDI levels, pg/mL	
Median	330
Mean	539.4
25% percentile	95
75% percentile	918

Data represent mean±SEM unless specified otherwise. HDL: High-density lipoprotein, LDL: Low-density lipoprotein.

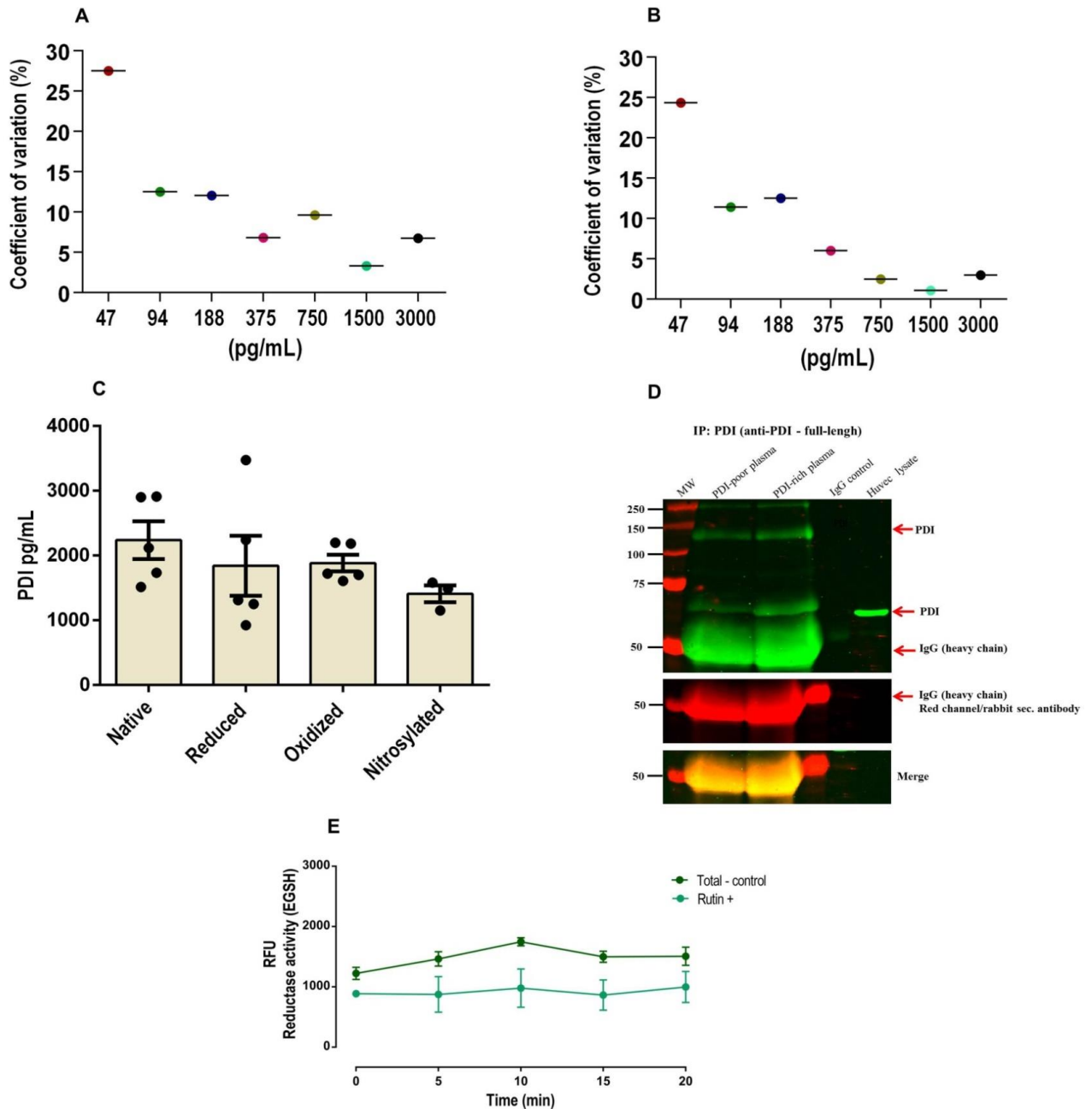
Table 2. Clinical characteristics of a population from a plasma databank who had cardiovascular events

<i>n</i>	95
Age (years)	50.4 ± 1.39
Male sex (%)	40
Total cholesterol, mg/dL	279.3 ± 7.5
HDL cholesterol	48.6 ± 2.3
LDL cholesterol, mg/dL	204.8 ± 7.3
Triglycerides, mg/dL	156.4 ± 9.2
Medical history (%)	
Hypertension	80
Diabetes	28
FH	35
Obesity	14
Type of CVE (%)	
Unstable angina	74
AMI	70
CVA	10
PDI levels, pg/mL	
Median	35
Mean	285.1
25% percentile	0
75% percentile	277

Data represent mean±SEM unless specified otherwise. HDL: High-density lipoproteins, LDL: Low-density lipoprotein, FH: familial hypercholesterolemia, CVE: Cardiovascular event, AMI: acute myocardial infarction, CVA: cerebrovascular accident.

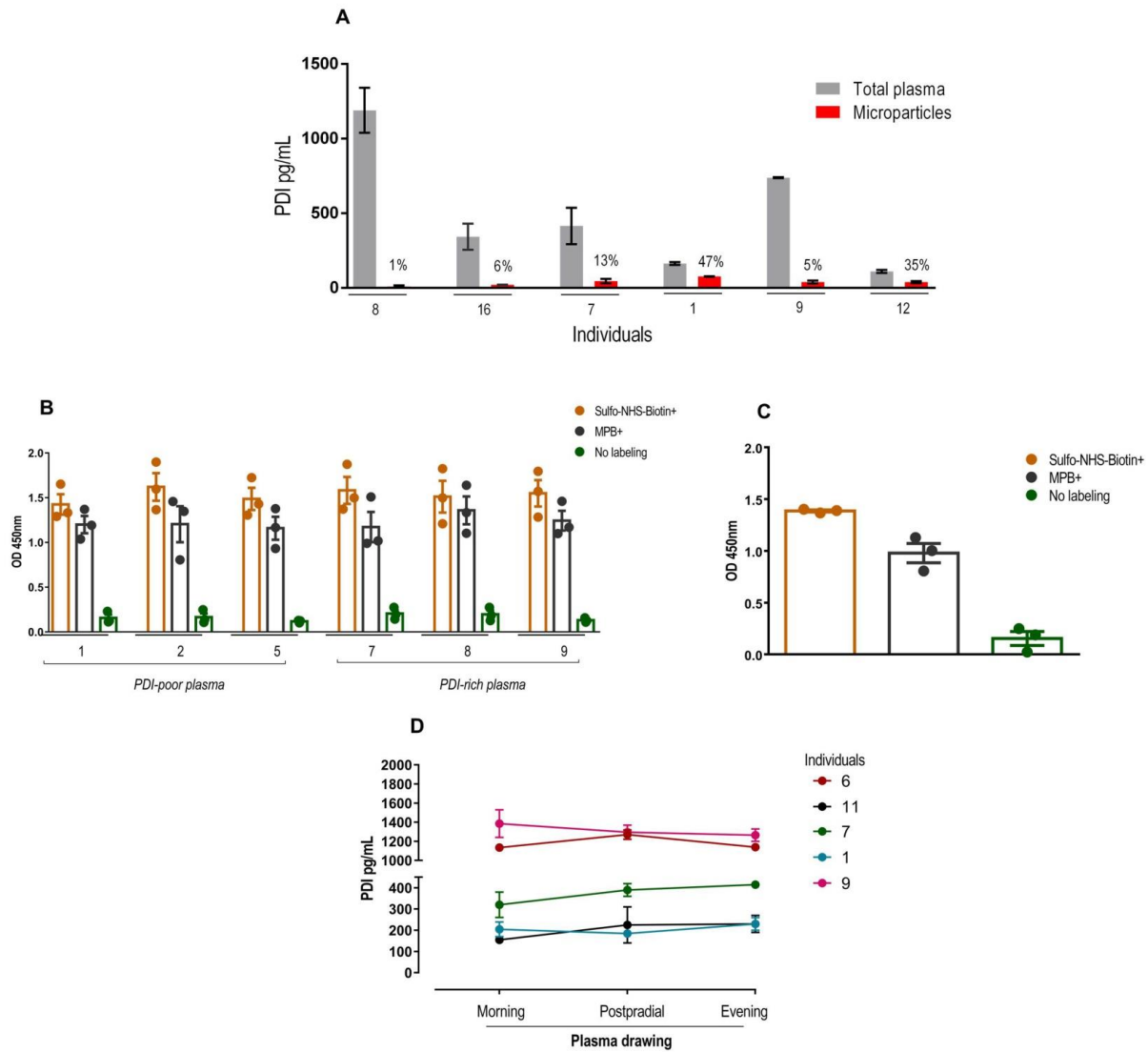
SUPPLEMENTARY MATERIAL

Figures

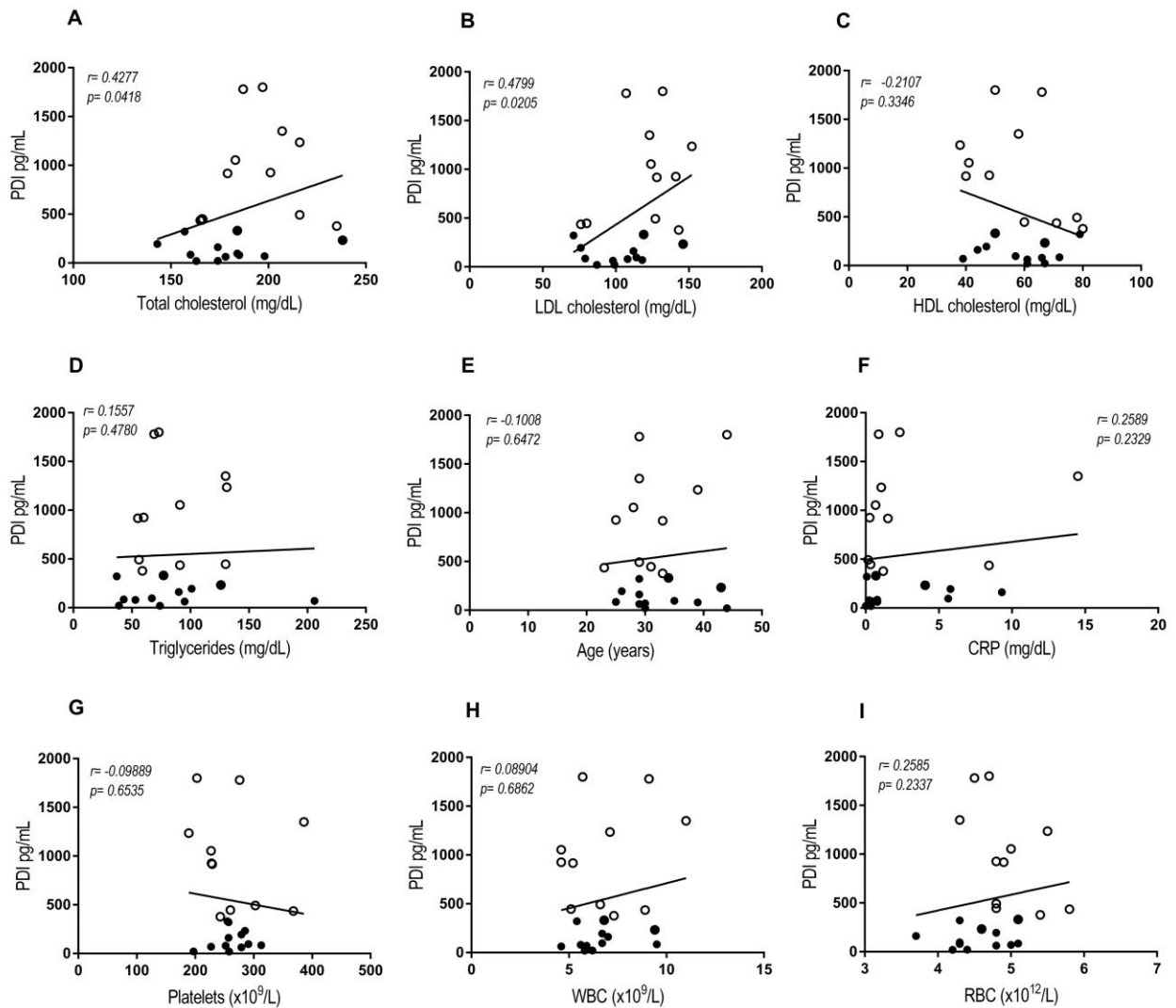


Supplementary Figure S1. Validation experiments for plasma PDI detection assay. A. Inter-assay precision was obtained by determining the coefficient of variation between duplicate samples obtained from the standard curves of ELISA assays, performed in separate occasions. PDI concentration varied from 47 to 3000 pg/mL. Log/log curve-fit was employed to calculate the concentration of PDI. Data from 4 independent experiments. **B.** Intra-assay precision was obtained

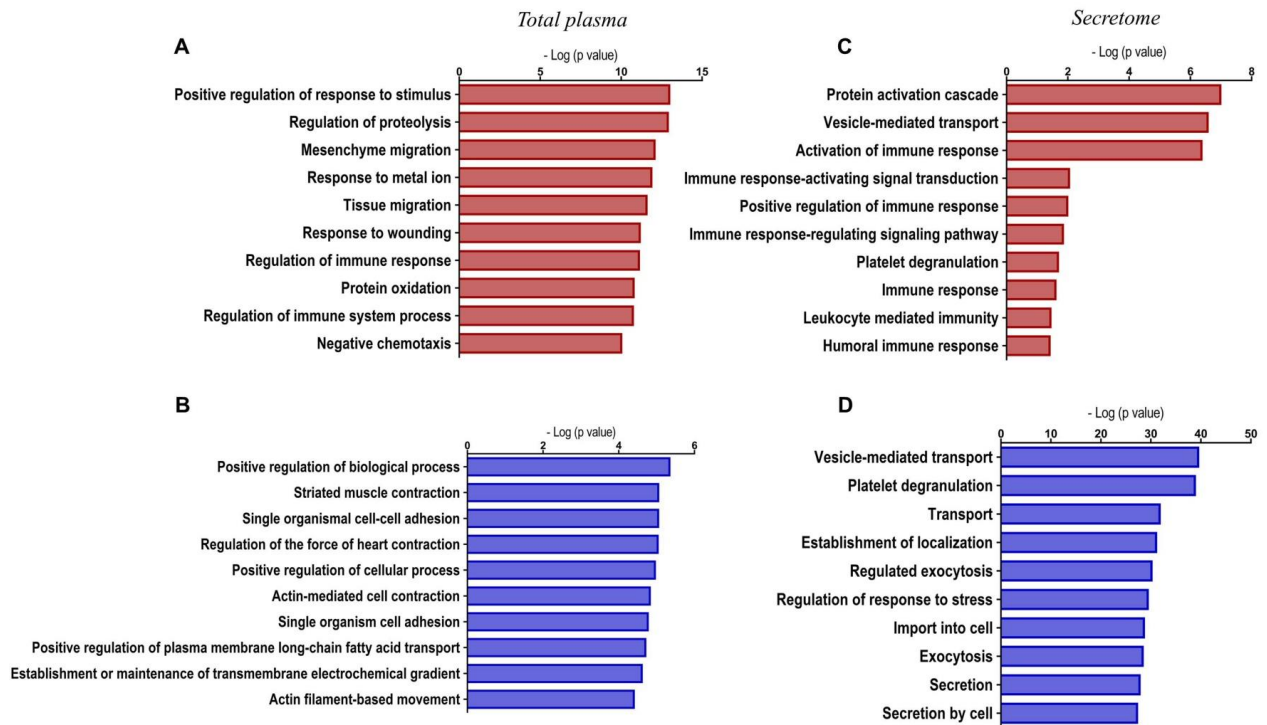
by determining the coefficient of variation between duplicate samples obtained from standard curves on the same plate measured by ELISA. PDI concentration varied from 47 to 3000 pg/mL. Log/log curve-fit was employed to calculate the concentration of PDI. Data from 4 independent experiments. **C.** Native, reduced, oxidized and S-nitrosylated PDI (2 ng/mL) was measured using ELISA (Human P4HB ELISA Par Set) on separate assays. Recombinant PDI was purified and reduced by GSH (10 mM), oxidized by diamide (10 mM) and nitrosylated by S-nitrosoglutathione (200-fold molar excess). Data represent mean \pm SEM from 3–5 independent experiments. No difference was detected among groups (One-way ANOVA followed by Tukey's post test). **D.** Uncropped western blots from PDI plasma immunoprecipitation to show the lack of cross-reaction between PDI band and IgG heavy chain. PDI bands in green (55 kDa and ~150 kDa). Controls show IgG heavy chain band location, in green channel (50 kDa), in red channel (50 kDa) and in yellow (merge between green anti-mouse and red anti-rabbit channels). Plasma PDI was immunoprecipitated with anti-PDI (rabbit IgG), the epitope is the full-length protein. Western blots: Anti-PDI (clone RL90, IgG mouse). Green and red channels are anti-mouse and anti-rabbit IgG secondary antibodies, respectively. **E.** Curve representing plasma PDI activity. Detection of plasma PDI reductase activity (di-eosin-GSSG assay) through formation of the reduced fluorogenic product eosin-5-isothiocyanate-coupled reduced glutathione (EGSH). Reductase activity was measured in platelet-poor plasma exposed or not to 60 μ M of rutin for 20 min. Data represent mean \pm SEM from 5 independent experiments. RFU, relative fluorescence units.



Supplementary Figure S2. Characteristics of plasma PDI. **A.** Detection of PDI in microparticles isolated from platelet-poor plasma of healthy individuals. Plasma microparticle fraction was obtained after multiple ultracentrifugations (as described in Materials and Methods). Thereafter, pellets were re-suspended in PBS and lysed for PDI measurement by ELISA. Data represent mean \pm SEM from 3 independent experiments. **B.** Detection of PDI redox state in human plasma. Plasma samples labelled with either EZ-Link Sulfo-NHS-Biotin (4 mM) or MPB (4 mM) were acetone-precipitated (to remove unbounded biotinylated probes) and protein pellets were re-suspended in PBS-tween (0.05%). Non-labelled samples incubated only with buffer were also carried out. Samples were added to a plate coated with anti-PDI antibody, probed with a monoclonal antibody to biotin (peroxidase conjugate) and absorbance was measured at 450 nm. Bar graph represents the optic density (OD) of each sample. Numbers represent different individuals. Data represent mean \pm SEM from 3 independent experiments. **C.** Pooled plasma sample derived from 3 individuals was used as an internal control and standard for each biotinylation assay to avoid any variation in MPB or EZ-Link Sulpho-NHS biotin labelling. Bar graph represents the optic density (OD) of each sample. Data represent mean \pm SEM from 3 independent experiments. **D.** Intra-individual variability in plasma PDI concentration along time. Plasma samples were obtained in three periods over the day: in the morning (fasting), postprandial and during the evening. Plasma PDI was measured by ELISA. Data represent mean \pm SEM from 3 independent experiments.

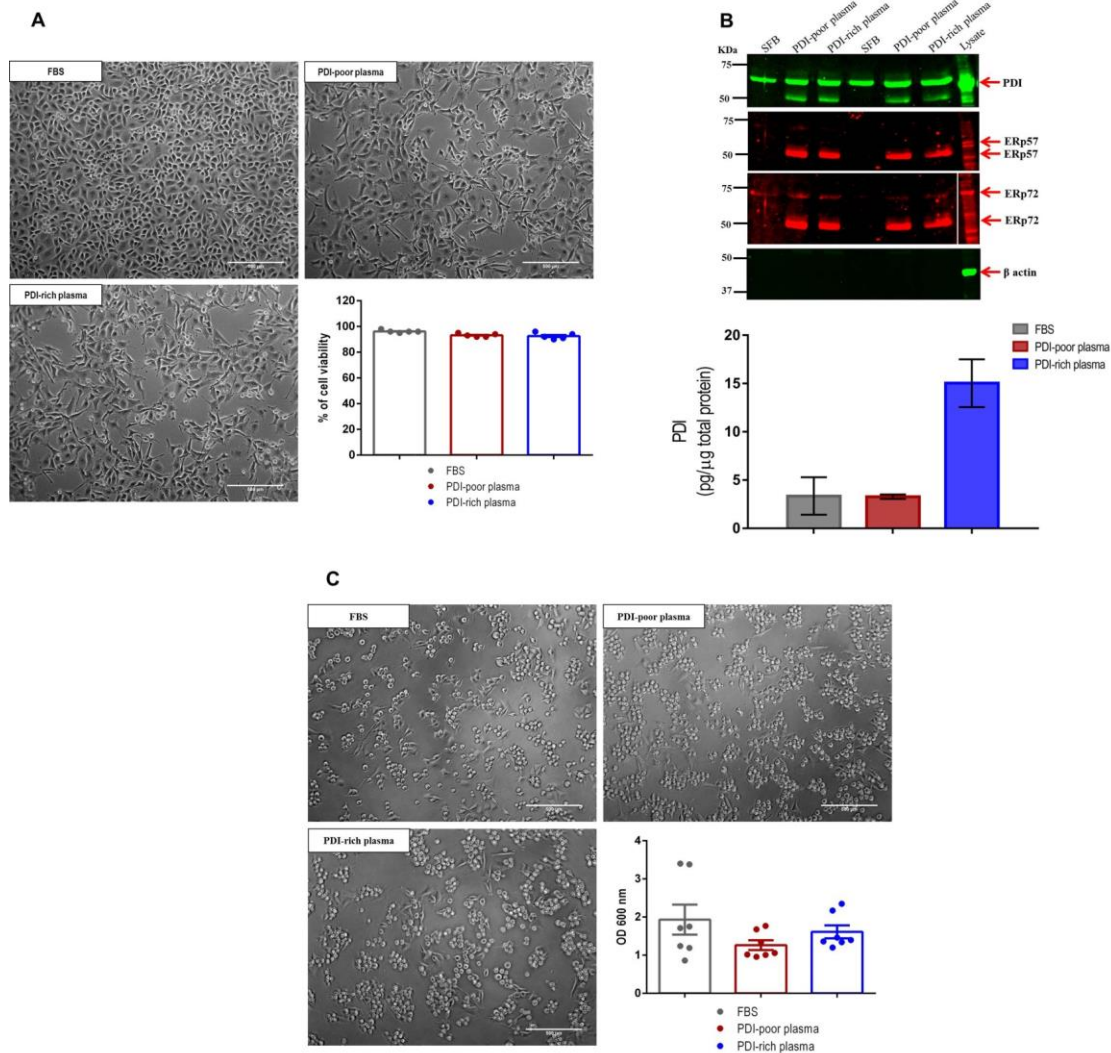


Supplementary Figure S3. Correlation between plasma PDI levels and different variables. (A) Total cholesterol levels, (B) Low-density lipoprotein (LDL) cholesterol levels, (C) High-density lipoprotein (HDL) cholesterol levels, (D) Triglyceride levels, (E) Age, (F) C-reactive protein levels, (G) Platelet count, (H) White blood cell count (I) Red blood cell count. All variables were plotted against plasma PDI concentration. Filled and open symbols represent individuals with PDI-poor and PDI-rich plasma, respectively ($n=23$). Correlation coefficient (r) and p -value are according to the method of Spearman; $p<0.05$ were considered significant.

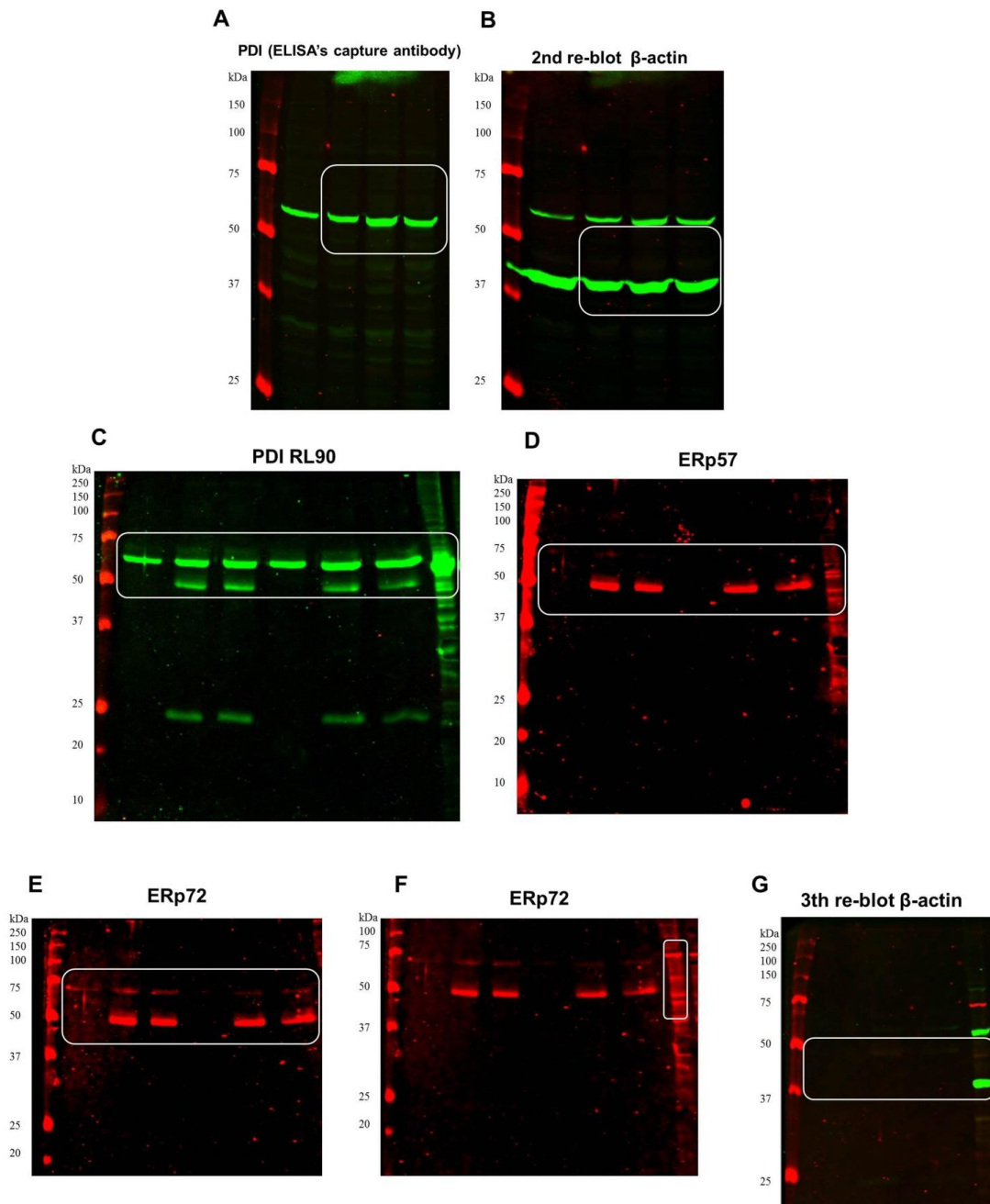


Supplementary Figure S4. Gene ontology process of proteins identified in proteome analysis.

A-B. Upregulated proteins differentially expressed between individuals with PDI-poor ($n=3$) and PDI-rich ($n=3$) plasma identified in all samples through shotgun mass spectrometry profiling approach were significantly enriched in several gene ontology process ($p<0.05$), analyzed by Metacore software. **C-D.** EC were incubated with 5% PDI-poor plasma, PDI-rich plasma or FBS for 24h and conditioned medium for secretome analysis was generated after additional 8h in serum-free media, as described in Materials and Methods. The subsets of upregulated proteins differentially expressed between EC exposed to either PDI-poor or PDI-rich plasma vs. FBS-control through shotgun mass spectrometry profiling approach were significantly enriched in several gene ontology processes ($p<0.05$), analyzed by Metacore software. All data are from three independent experiments. PDI-poor plasma: red bars. PDI-rich plasma (blue bars).



Supplementary Figure S5. Effects of endothelial cell incubation with PDI-poor or PDI-rich plasma. **A.** Cell viability was determined by Trypan Blue exclusion assay. Photomicrographs represent cells after 24h of incubation with PDI-poor plasma, PDI-rich plasma or FBS-control. Bars graph represents percent cell viability. Data represent mean \pm SEM from 5 independent experiments. No difference was detected among groups (One-way ANOVA with Tukey's post test). **B.** Protein secretome validation. Western blot analysis of conditioned medium (CM) from HUVEC incubated with PDI-poor plasma, PDI-rich plasma or FBS-control. Samples were submitted to SDS-PAGE and immunoblotted with anti-PDI, anti-ERp57, anti-ER72 and anti- β actin antibodies. Importantly, there was no evidence of cell damage within the secretome, since there was no detectable β -actin in the CM. Representative of 3 independent experiments. Uncropped western blots are shown in Supplementary Fig.S6. ELISA assay further confirmed the presence of PDI in secretome samples. Data represent mean \pm SEM from 3 independent experiments. **C.** HUVEC were pre-incubated with 5% PDI-poor plasma, PDI-rich plasma or FBS for 24h, seeded (4×10^4 cells/mL) in 96-well plates coated with collagen-1 (10 μ g/mL) and allowed to adhere for 4h30min at 37°C. After washing, adherent cells were stained with crystal violet, solubilized by SDS and absorbance was measured at 600 nm. Scale bar: 500 μ m. Objective: 40x. Data represent mean \pm SEM from 5 independent experiments. No difference was detected among groups (One-way ANOVA with Tukey's post test).



Supplementary Figure S6. Uncropped Western Blots. **A.** Western blot against PDI in human cell lysates (HUVEC, HCT116, HKE3). Representative of 3 independent experiments (from Fig.1C). **B.** Western blot against β -actin in human cell lysates (HUVEC, HCT116, HKE3). Representative of 3 independent experiments (from Fig.1C). **C.** Western blot against PDI in EC conditioned medium (CM) and lysate. Representative of 9 samples from three independent experiments (From Supplementary Fig.S5-B). **D.** Western blot against ERp57 in EC CM and lysate. Representative of 9 samples from three independent experiments (From Supplementary Fig.S5-B). **E.** Western blot against ERp72 in EC CM. Representative of 9 samples from three independent experiments (From Supplementary Fig.S5-B). **F.** Western blot against PDI in EC lysate. Representative of 9 samples from three independent experiments (From Supplementary Fig.S5-B). **G.** Western blot against β -actin in EC CM and lysate. Representative of 9 samples from three independent experiments (From Supplementary Fig.S5-B).

Tables

Supplementary Table S1. Upregulated proteins differentially expressed in total plasma proteome from individual with PDI-poor plasma

Supplementary Table S2. Upregulated proteins differentially expressed in total plasma proteome from individual with PDI-rich plasma

Supplementary Table S3. Gene list RT² profiler PCR array

Supplementary Table S4. Upregulated proteins differentially expressed in secretome samples from endothelial cells incubated with PDI-poor plasma vs. controls (FBS)

Supplementary Table S5. Upregulated proteins differentially expressed in secretome samples from endothelial cells incubated with PDI-rich plasma vs. controls (FBS)

Supplementary Table S6. Association between plasma PDI levels and traditional risk factors in patients with cardiovascular events (databank population).

Apêndice B

Artigo 2: *Implications of plasma thiol redox in disease. Clinical Science.* 2018; doi:
10.1042/CS20180157 (revisão).

Review Article

Implications of plasma thiol redox in disease

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Thiol groups are crucially involved in signaling/homeostasis through oxidation, reduction, and disulphide exchange. The overall thiol pool is the resultant of several individual pools of small compounds (e.g. cysteine), peptides (e.g. glutathione), and thiol proteins (e.g. thioredoxin (Trx)), which are not in equilibrium and present specific oxidized/reduced ratios. This review addresses mechanisms and implications of circulating plasma thiol/disulphide redox pools, which are involved in several physiologic processes and explored as disease biomarkers. Thiol pools are regulated by mechanisms linked to their intrinsic reactivity against oxidants, concentration of antioxidants, thiol-disulphide exchange rates, and their dynamic release/removal from plasma. Major thiol couples determining plasma redox potential (E_H) are reduced cysteine (CyS)/cystine (the disulphide form of cysteine) (CySS), followed by GSH/disulphide-oxidized glutathione (GSSG). Hydrogen peroxide and hypohalous acids are the main plasma oxidants, while water-soluble and lipid-soluble small molecules are the main antioxidants. The thiol proteome and thiol-oxidoreductases are emerging investigative areas given their specific disease-related responses (e.g. protein disulphide isomerases (PDIs) in thrombosis). Plasma cysteine and glutathione redox couples exhibit pro-oxidant changes directly correlated with ageing/age-related diseases. We further discuss changes in thiol-disulphide redox state in specific groups of diseases: cardiovascular, cancer, and neurodegenerative. These results indicate association with the disease states, although not yet clear-cut to yield specific biomarkers. We also highlight mechanisms whereby thiol pools affect atherosclerosis pathophysiology. Overall, it is unlikely that a single measurement provides global assessment of plasma oxidative stress. Rather, assessment of individual thiol pools and thiol-proteins specific to any given condition has more solid and logical perspective to yield novel relevant information on disease risk and prognosis.

Introduction

Thiol or sulphhydryl ($-SH$) is a highly active and versatile form of reduced sulphur in biomolecules. It is present in amino acids such as cysteine (Cys) in peptides and proteins and is particularly sensitive to redox reactions, capable of acting as a crucial redox sensor as well as a switch able to post-translationally modify protein function and interactivity. As such, thiols undergo an array of redox reactions, in particular with oxidants and disulphides, which depend mainly on species type and concentration (Figure 1) [1,2]. Generated products such as disulphides and intermediate states of thiol oxidation such as sulphenic acid can revert to thiols by reductants, while sulphinic acid can be reduced enzymatically [3] and sulphonic acid is believed to be a final irreversible product [4]. Intramolecular disulphides are essential for protein folding and structure and can crucially regulate protein function [5]. Meanwhile, intermolecular disulphides may account for functional protein switches (e.g. following glutathionylation) as well as protein oligomerization and complex formation. An important type of thiol reactivity, of relevance to the present article, is thiol-disulphide exchange, a reaction involving the nucleophilic attack of an ionized thiolate (RS^-), mainly from thiols with a low acid dissociation constant (pK_a), on an existing disulphide bond, resulting in the

Received: 14 February 2018
Revised: 09 May 2018
Accepted: 22 May 2018

Version of Record published:
29 June 2018

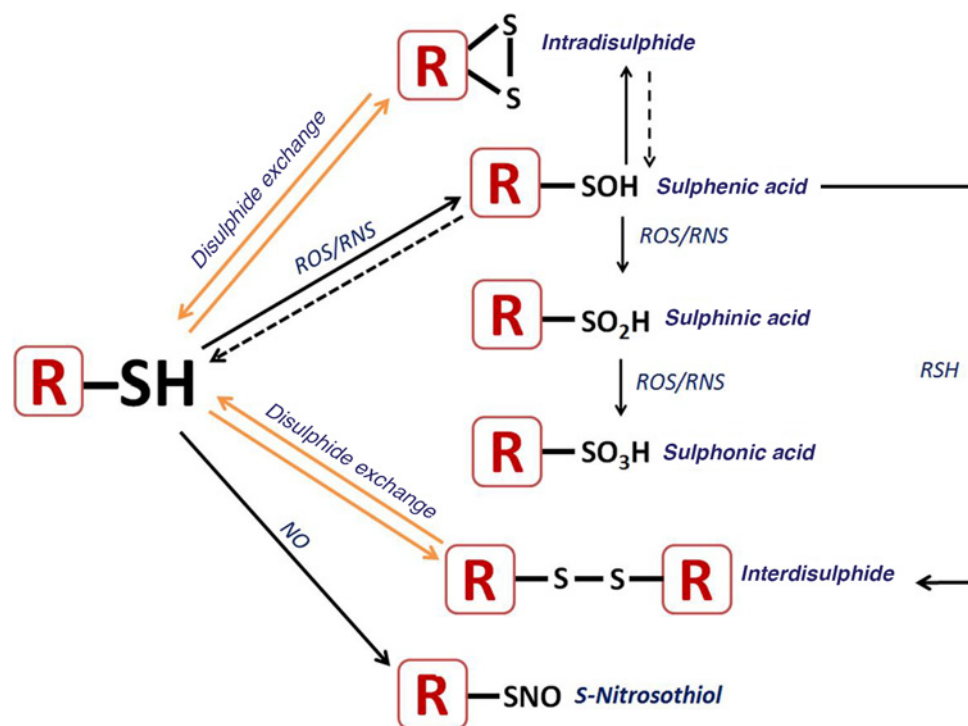


Figure 1. Array of thiol reactivity

Thiols can generate a diverse array of modifications, including reversible ones, such as intra and interdisulphides, S-sulphenation, S-sulphination, S-nitrosation, and also non-reversible modifications such as S-sulphonation (adapted from Chen et al. (2003) [2]). The full range of these modifications is much higher but here we only highlighted the main pathways, which are those discussed in the text.

exchange of a covalent disulphide bond (i.e. the electron pair rather than single electrons as in radical reactions). Disulphide formation and breakdown depends mainly on kinetic factors, but also on the availability of electron donors or acceptors, which determine the overall ambient redox potential (E_h) [6]. The E_h for an oxidation/reduction couple (e.g. reduced cysteine (CyS)/cystine (the disulphide form of cysteine) (CySS)) is dependent upon the inherent thermodynamic tendency of the chemical species to accept/donate electrons (i.e. the reduction potential) and the concentrations of the respective acceptors and donors [7]. The reduction potential is quantitatively expressed in millivolts (mV) relative to the standard hydrogen electrode, on a scale of positive (more oxidizing) to negative (more reductive) values. For example, the reduction potential for the highly oxidizing hydroxyl radical ($\text{HO}\bullet$) is +2310 mV and for the highly reductive glutathionyl anion radical ($\text{GSSG}^{\bullet-}$) -1500 mV, while hydrogen peroxide (H_2O_2) and superoxide reduction potentials are approximately +320 or -330 mV, respectively, in biological systems [8]. Redox potentials allow predictions concerning reactions amongst different biochemicals but tell little about the kinetics of such reactions [9,10].

The main thiol compounds of biological interest (Table 1) are: (i) low molecular weight (LMW) small compounds such as cysteine, acetyl-CoA, cysteamine; (ii) LMW peptides such as glutathione; (iii) thiol proteins, that can be arbitrarily classified as: thiol-regulated signaling proteins (kinases, phosphatases, transcription factors), which are thiol targets in the context of signal transduction regulation; thiol-storage proteins (albumin); professional thiol redox proteins (glutathione peroxidases (GPx), peroxiredoxins (Prdx), glutathione transferases, thioredoxins (Trx), protein disulphide isomerases (PDIs)), which comprise those proteins directly involved in thiol redox regulation upstream to the targets of cell signaling.

An important feature of thiol systems is their compartmentalization. In general, thiol-containing molecules react widely amongst themselves, forming a true thiol redox network, essential for a host of cellular functions. However, the main individual thiol redox pairs by themselves do not appear to be in equilibrium [7], indicating that such thiol redox network can be approached as a resultant of several specific thiol pools, each with a given redox state. This fact reflects the subcellular compartmentalization of thiol pools and the respective oxidant/antioxidant levels, redox

Table 1 Main thiol compounds of biological/biomedical relevance

Class	Type	Examples
LMW thiols	Small compounds	Cysteine Cysteamine Acetyl-CoA Hydrogen sulphide
	Peptides	Glutathione Cysteinyglycine
Thiol proteins	Thiol-regulated signaling proteins	Kinases Phosphatases Transcription factors
	Thiol-storage proteins	Albumin
	Professional thiol redox proteins	GPx Prdx Glutathione transferases Trx PDIs

routes, and kinetic constraints, together with the biological processes in which they participate. Accordingly, cellular subcompartments differ in their redox poise so that their thiol/disulphide redox couples within organelles are maintained at distinct steady-state redox potentials and respond separately to physiologic signaling. This is particularly evident for the extracellular, as compared with intracellular, thiol pools, which may differ in their respective concentrations by 100-fold or more (Figure 2 and section *Thiol/disulphide pools in human plasma*). How the extracellular thiol pools cross-talk with the intracellular ones is yet poorly known. Although the secretion and uptake of thiols and disulphides is well known, the underlying pathways are unclear. Some enzymatic membrane transport systems have been well studied in specific settings, such as those for glutathione [11,12], as well as cysteine [13,14]. However, in eukaryotes, the existence and functional roles of transmembrane electron-transporting thiol proteins analogous to the prokaryote DsbD β [15] remains unknown.

A particular extracellular thiol/disulphide pool, of great biomedical significance, is the plasma pool, which participates in several processes such as cell signaling, antioxidant defense, metal complexing, protein regulation, macromolecular structure and trafficking [4,9]. The fact that distinct thiol pools in biological systems are not in equilibrium also extends to the plasma compartment and raises some implications. First, this indicates that the concept of oxidative stress as a global shift in the balance of pro-oxidants and antioxidants has significant limitations. Second, it follows that plasma oxidative stress cannot be approached only from a single thiol redox pair because multiple systems are involved on redox signaling and these systems do not have the same sensitivity to oxidants or respond equally to antioxidants [16]. In parallel, quantitative analysis of thiol redox state of plasma can provide relevant information as a clinical evidence of oxidative burden *in vivo*, such as for the cysteine (reduced)/cystine (oxidized) pair [9,16,17]. Also, a number of studies have shown that LMW thiol-disulphide pool in human plasma can be related with oxidative reactions and antioxidant defenses in several disease conditions (see section II). However, the complexity and multiple factors associated with thiol pools in plasma pose a challenge with respect to drawing clear-cut conclusions about their possible roles as disease markers. In this review, we discuss mechanistic foundations underlying the plasma thiol pools in perspective with their possible implications in disease states.

Thiol/disulphide pools in human plasma

Thiol pools and their concentrations: intracellular compared with plasma

Thiol pools are distinctly segregated in intra compared with extracellular compartments (Figure 2). In mammals, the most abundant LMW thiol pool is glutathione, with a total concentration in the millimolar range (e.g. approximately 10 mM in liver) [18], >90% in the reduced state (GSH) within cells [19]. Intracellular GSH/disulphide-oxidized glutathione (GSSG) redox state varies in association with proliferation, differentiation, and apoptosis [20]. Conversely, in plasma, glutathione is present at micromolar concentrations [9], as further discussed below. Another important LMW thiol pool is cysteine (Cys). Cys levels in cells are much lower than glutathione, in the range of 50–150 μ M. Despite this, Cys levels can account for the rate of glutathione synthesis, although they can also vary irrespective of GSH [21]. On the other hand, in human plasma Cys is the major LMW thiol and maintains interorgan glutathione balance [22] and protein synthesis [23]. Protein thiols are more abundant than glutathione, often in the 10–50 mM range, representing approximately 70% of total intracellular pool of reduced thiols [19]. Disulphide concentration frequently

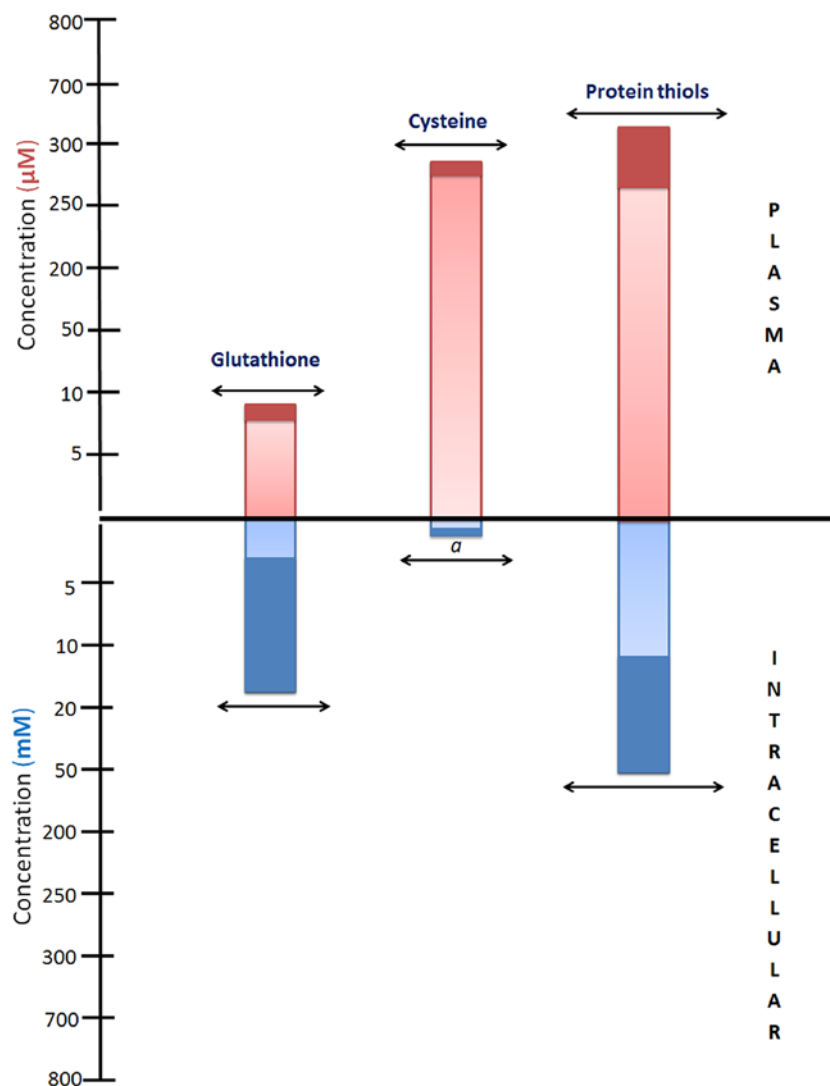


Figure 2. Comparison between the main thiols pools regarding plasma compared with intracellular concentrations

Data are presented in a natural logarithmic scale. Please note that the values are expressed as micromolar concentrations for plasma thiols compared with millimolar levels for intracellular thiols. Because of the ln scale, values for intracellular cysteine, which are quite low, are not proportionally represented. The plasma concentration range for glutathione, cysteine, and protein thiols are (in μM): 4.9–7.3, 202–281, and 200–500, respectively. The intracellular concentration range for glutathione, cysteine, and protein thiols are (in mM): 2–17, $5\text{--}15 \times 10^{-4}$, and 10–50, respectively. The range of values is represented by the dark bars.

varies in association with changes in corresponding thiols in biologic systems, however disulphide concentrations are not completely consistent with the expected stoichiometry for thiol oxidation to disulphide.

Plasma thiol/disulphide redox pools are highly regulated. Figure 3 provides a summary of the major thiol pools in human plasma and their relative concentrations. The total glutathione concentration in human plasma is 4.9–7.3 μM , while the concentrations of GSH and GSSG are, respectively, 2.0–5.1 and 0.7–1.9 μM , which, as discussed above, are <1% of their tissue levels [24–27]. In parallel, the values for plasma GSH/GSSG E_h are approximately -137 ± 9 mV (mean: -140 mV) in young healthy individuals, considerably more oxidizing (by an average of 90 mV) than the cytoplasmic pool (-185 to -258 mV in tissues and cultured cells) [16,28]. Extracellular release of GSH or GSSG is a function of their respective cell concentrations, so that intracellular increase in GSSG levels or decrease in GSH levels promote, respectively, higher GSSG or lower GSH efflux into plasma [16].

The major thiol/disulphide redox pool in mammalian plasma is the CyS/CySS couple [28]. Total concentration in serum/plasma from healthy subjects is approximately 202–281 μM , of which approximately 65% is protein-bound,

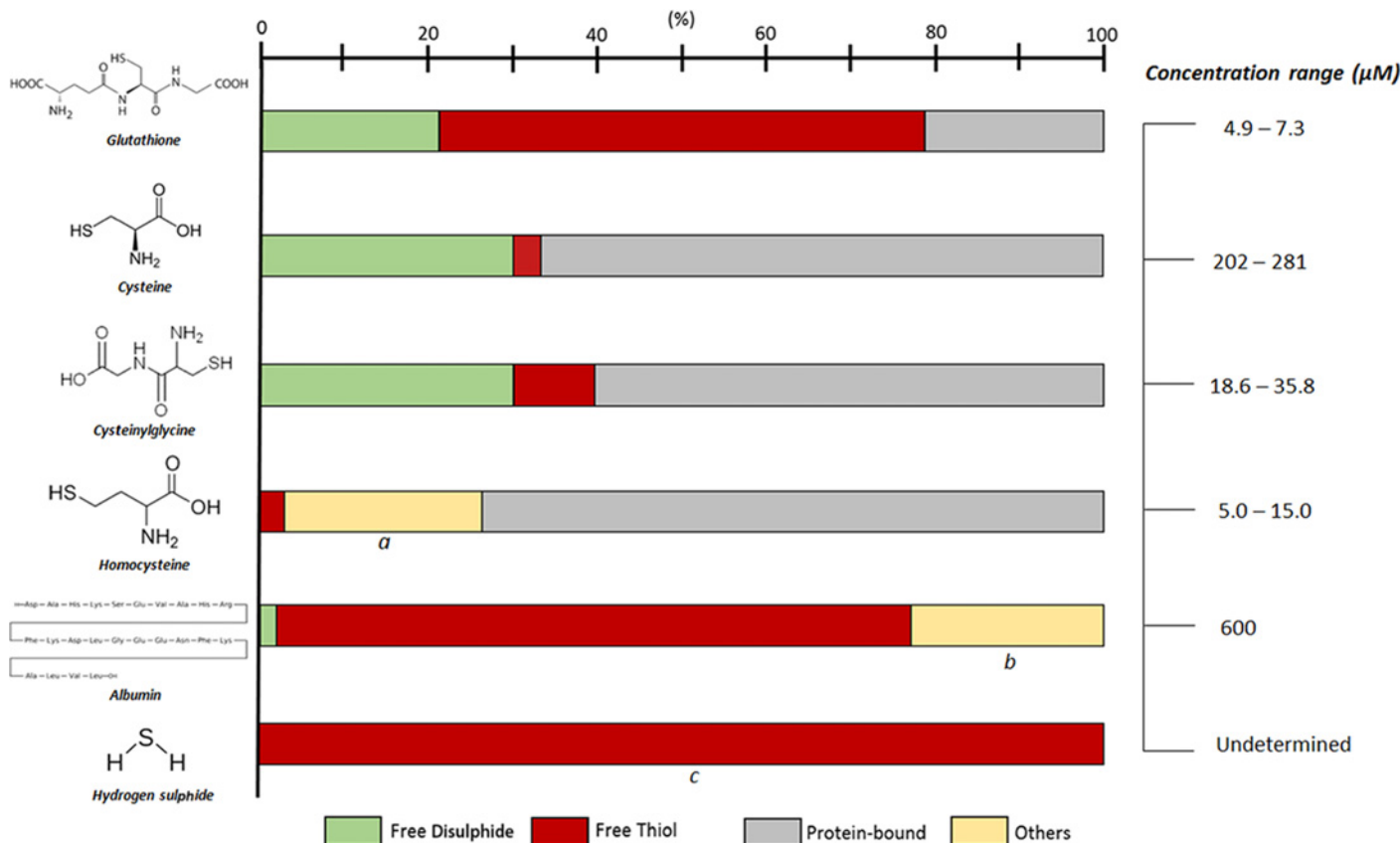


Figure 3. The main thiol redox couples and their concentrations in human plasma

^a Dimer; ^b human serum albumin (HSA) mixed disulphides with proteins and LMW thiols; ^c estimated yield values (divergent information).

30% is free-oxidized, and 3–4% is reduced [24–26,29]. CySS concentrations (40–50 μM) are higher than CyS (8–10 μM) and the E_h of the CyS/CySS couple is -80 ± 9 mV (young healthy individuals), which is approximately 57 mV more oxidizing than that of GSH/GSSG pool. Additionally, Cys is more reactive than GSH in thiol-disulphide exchange with plasma proteins [24,28,30].

Regarding other LMW plasma thiols, the concentration of cysteinylglycine (Cys-Gly), with total levels of 18.6–35.8 μM, is considerably lower than that of Cys but still higher than glutathione and Cys-Gly is approximately 60% protein-bound, 30% free-oxidized, and 10% reduced [24–26]. Homocysteine (Hcy) depicts different forms in plasma: approximately 1–3% circulates as free thiol, 70–80% remains bound as mixed disulphide to plasma proteins, mainly albumin, and 20–30% combines with itself to form Hcy homodimers or heterodimers with other thiols [31]. In healthy humans, normal fasting levels are low, ranging from 5 to 15 μM as assessed by HPLC or 5 to 12 μM by immunoassay [32]. Human serum albumin (HSA), the most abundant thiol in plasma (concentration: approximately 43 g/l = approximately 0.6 mM), is predominantly (approximately 75%) reduced [4], while approximately 1–2% of total HSA exhibits their thiols oxidized to higher oxidation states such as sulphinic or sulphonic acids [33] (see Figure 1). Total concentration of γ -glutamylcysteine, the immediate glutathione precursor, is 3–5 μM, and it is approximately 1% reduced [26,34]. More recently, significant attention has been driven to hydrogen sulphide (H_2S/HS^-), the smallest member of the thiol family. H_2S/HS^- has traditionally been considered a toxic product, however this has changed following the discovery of its synthesis routes in mammals, as well as a number of associated physiological, as well as pathological effects [4]. The plasma concentrations of H_2S are not clear: while some studies have shown concentrations of 30 to >100 μM [35–38], another study found values of 1×10^{-4} μM [39].

Thiol-disulphide equilibrium in plasma

The thiol pools discussed above have general antioxidant properties, composing a collective effect as intracellular redox buffer, regulating protein thiol/disulphide composition via thiol-disulphide exchange (Figure 1). These, in turn,

depend on a number of kinetic and thermodynamic variables, which will be discussed here in more detail [40]. As already commented, plasma thiol/disulphide couples (especially CyS/CySS and GSH/GSSG) are under non-equilibrium state, although there is significant exchange of thiol equivalents. This design allows dynamic and rapid regulation, supports redox signaling and acts as a central target of non-radical mechanisms of oxidative burden [41]. The general exchange reaction between thiols and disulphides is represented by:



The factors beyond mutual equilibration affecting actual levels of reduced and oxidized thiol pools include (discussed by Turell et al. (2013) [4]): (i) rates of thiol-disulphide exchange reactions; (ii) thiol oxidation rates by reactive oxygen species (ROS) and possible repair processes; (iii) rates of enzymatic extracellular glutathione degradation; (iv) rates of transport between plasma and cell, particularly erythrocytes and endothelial cells; (v) release rates of thiol-containing molecules such as HSA and glutathione, mainly by the liver and, to a minor extent, rates of renal excretion; and (vi) intracellular metabolism. The contribution of these different mechanisms to the maintenance of steady-state levels of each thiol pool is associated with their varied kinetic elimination from plasma. For example, the disappearance of CyS and Cys-Gly is slower than that of glutathione and Hcy [42]. This effect is seen, e.g. upon chemotherapy, with depletion of GSH and Hcy occurring in the first place, while Cys depletion occurs with a time lag of approximately 2 h after the end of chemotherapeutic infusion [43].

Another way to address these mechanisms is to follow the sequential changes after exogenous intravenous administration of a reduced thiol. GSH infusion increases both total glutathione and Cys plasma levels, followed by their elimination within minutes. However, the rate of GSH disappearance is substantially faster than that of total glutathione, probably reflecting mechanisms such as metabolization by γ -glutamyl transferase and formation of mixed disulphides. The increase in CyS plasma levels likely associates with the reduction in circulating CySS or cysteine-mixed disulphides by high GSH levels. However, the total cysteine concentration transiently decreases, probably due to its enhanced cellular uptake. Subsequently, total Cys plasma levels increase, probably reflecting their cellular release either as Cys or glutathione, which is catabolized to cysteine [30]. Other studies with intravenous administration of reduced Hcy indicated that increased plasma levels of reduced Hcy peaked approximately 1 min prior to peak concentrations of CyS and Cys-Gly [44]. These approaches highlight the complex mechanisms involved in the interplay of plasma thiol redox control systems upon a physiological thiol load. A clinically relevant situation is the exogenous administration of the common thiol drug *N*-acetylcysteine (NAC). Intravenous administration of NAC transiently increases plasma thiol concentrations, mainly by altering the pool of protein-bound thiols through a thiol-disulphide exchange reaction [4]. Moreover, oral NAC treatment significantly decreases total plasma Hcy concentration [45]. NAC has been widely used as a treatment of acetaminophen toxicity-associated liver injury, since NAC supplies the cysteine required for *de novo* synthesis of hepatic GSH, helping to restore its levels [46]. However, the rapid antioxidant effects of NAC, well-known especially in *in vitro* systems, cannot be explained by these effects, nor by NAC scavenging of oxidants. Rather, a recent study disclosed a novel mechanism, the mitochondrial production of sulphanes from NAC-triggered H₂S [47].

LMW oxidant levels in plasma

Any discussion of plasma thiol redox state has to take into account the parallel levels of oxidants, particularly LMW oxidants including H₂O₂, hypochlorous acid (HOCl) and peroxynitrite (ONOO⁻), amongst others. Their concentrations in plasma compartment are not very clear because there is no obvious steady state and their levels vary according to many (patho) physiological conditions, as well as with technical interferences. However, the available information in the literature indicates that the overall concentration of LMW oxidants in plasma is generally low.

H₂O₂ plasma concentrations are 100–5000 times larger than intracellular ones, although such plasma levels are not at a steady state and are dynamically variable. From analysis of literature and estimated kinetics, the probable normal range for plasma H₂O₂ is 1–5 μ M, however it can rise as high as 50 μ M in inflammatory diseases. However, the precise values remain ambiguous given the high variability of published results, mainly because of questionable measurement techniques [48]. The main sources of plasma H₂O₂ appear to be the plasma membrane NOX NADPH oxidase complexes of phagocytes, endothelial cells, and platelets [49], with possible additional contributions of xanthine oxidase. The latter is released into the circulation primarily from hepatocytes but also other cells, and is able to bind to the surface of endothelial cells, where it supports H₂O₂ production [50]. The autoxidation of small molecules, e.g. catecholamines, is another potential source of extracellular H₂O₂, however this process appears to be a minor contributor under physiological conditions.

In blood, ONOO^- reacts quite rapidly with carbon dioxide, which is in equilibrium with plasma bicarbonate [51]. This reaction forms a highly reactive short-lived secondary oxidant, the nitrosoperoxycarbonate adduct (ONOOCO_2^-), leading to the reactive intermediates $\text{NO}\bullet_2$ and $\text{CO}_3\bullet^-$, which in turn account for free or protein-bound tyrosine nitration, yielding the stable compound 3-nitro-tyrosine (3NT) [52,53]. This process has been linked to oxidative modifications of low-density lipoprotein (LDL) to a form which is recognized by scavenger receptors from macrophages [54]. Based on 3NT detection, involvement of ONOO^- in atherogenesis has been proposed [52,55].

The highly chlorinating and oxidizing intermediate HOCl is known to be generated mainly by myeloperoxidase (MPO) from phagocytes. Several studies have shown that GSH and protein thiols are preferred targets for HOCl [56]. However, regardless of its high reactivity, plasma GSH consumes only a small amount of inflammatory HOCl. It reacts most rapidly with Cys and methionine residues, while its reactivity with side chains bearing His, Trp, Lys, Arg and Tyr residues is two to five orders of lower magnitude.

Another potential oxidant source in plasma is vascular peroxidase 1 (VPO1), which is secreted from endothelial cells [57]. The estimated amount of VPO1 in human plasma may be approximately four orders of magnitude higher than that of MPO, in the range of $1.1 \pm 0.6 \mu\text{M}$ in healthy individuals. While the biological functions of circulating VPO1 in human plasma are yet unclear, it is likely that VPO1 also displays a peroxidase effect not unlike that of MPO and utilizes NOX-derived H_2O_2 to produce hypohalous acids including HOCl [58]. VPO1 has enzymatic properties characteristic of the peroxidase-cyclooxygenase family, except with regard to heat stability [57]. Additionally, VPO1 catalyzes collagen IV sulphilimine cross-linking, an oxidative post-translational modification critical for basement membrane integrity and tissue biogenesis [59].

Plasma antioxidants and thiol-disulphide equilibrium

The concepts discussed so far implicate the multilevel interaction between thiols and oxidants in plasma redox processes. This raises the importance of also discussing the involvement of plasma antioxidant species in such processes, although little is known regarding possible direct interactions between thiol pools and known plasma antioxidant systems. Despite the several antioxidant mechanisms present in plasma, which have been extensively studied and are briefly detailed below, this compartment cannot be considered a net antioxidant fluid. In fact, reduced LMW thiols are not at high concentrations and antioxidant proteins are scarce in plasma. In addition, some detected antioxidant effects of plasma derive from the contribution of erythrocytes [4,60].

The main antioxidant mechanisms in human plasma can be classified into two main groups: (i) water-soluble antioxidants, including ascorbic acid (AA) and uric acid (UA) and (2) lipid-soluble antioxidants associated with lipoproteins, including α - and γ -tocopherol, ubiquinol, lycopene, β -carotene, and other carotenoids and oxycarotenoids. In addition, plasma protein thiols and bilirubin also impart additional antioxidant functions [61]. These antioxidants work synergistically to regenerate each other as an 'antioxidant network' [62]. A classical example is GSH, which regenerates AA, which then regenerates α -tocopherol from its radical forms [63]. AA and ubiquinol behave as 'firstline' antioxidants in the aqueous and lipid phases of human plasma, respectively. These species are first consumed when plasma is exposed to oxidants and prevent oxidative modifications while available [62]. Thus, within such hierarchy, plasma antioxidant capacity associates with the interaction and co-operation of several antioxidants [64]. In addition, transferrin and ceruloplasmin are considered preventive plasma antioxidants because they sequester transition metals [65]. Furthermore, extracellular superoxide dismutase (EC-SOD), GPx, and HSA were also shown to be involved in antioxidant defenses in plasma.

AA or vitamin C is an important biological antioxidant capable of scavenging a broad oxidant variety. In human plasma, AA concentrations are 30–150 μM [61,66]. AA is able to prevent lipid hydroperoxide formation in plasma lipoproteins, e.g. LDL, at least in part by reducing α -tocopherol radicals formed upon reaction with lipid peroxy radicals [67,68]. Protective AA effects extend to cell membranes and even across them [69,70]. Despite the slow reaction between superoxide and AA, plasma AA concentrations detectably prevent H_2O_2 production in blood [48].

UA (the end product of purine metabolism in humans) is believed to account for approximately 60% of free-radical scavenging capacity of plasma [71]. UA plasma concentration is approximately 160–450 μM [4,61]. Such levels are influenced by food intake, e.g. high-fructose fruits promote increase in UA levels within the normal range, supporting plasma antioxidant capacity [72]. Elevated plasma UA levels, however, correlate with increased risk of cardiovascular disease (CVD) [73] and gout [74]. This has been mainly attributable to the effects of UA crystals, but a direct proinflammatory effect can also be responsible [75]. The interaction of UA and ONOO^- was shown to result in a thiol-independent nitrated product able to trigger NO release and endothelium-independent vasorelaxation [76].

Bilirubin, the end product of heme catabolism, has both cytotoxic and cytoprotective properties [77], the latter at least in part due to a major antioxidant role of bilirubin at physiological concentrations [78,79]. In human plasma, bilirubin is mainly bound to albumin at concentrations approximately 5–20 μM [4,61]. Elevated serum bilirubin is protective against atherosclerotic microvascular dysfunction [80] and associates with less severe peripheral artery disease [81,82]. These results are in line with the reported roles for bilirubin as free radical scavenger in distinct organs such as heart [83], neurones [84], kidney, liver, and gut [85,86].

The lipid-soluble antioxidants from plasma lipoproteins are α - and γ -tocopherol, lycopene, β -carotene, and ubiquinol, the reduced form of coenzyme Q₁₀ [4,61]. α and γ -tocopherol belong to the vitamin E family [87], while α -tocopherol corresponds to 95% of total vitamin E content [88]. α -tocopherol is particularly well known as a strong chain-breaking, peroxy radical-scavenging antioxidant [86], due to its 6-hydroxychroman structure. Its concentration in human plasma is approximately 15–40 μM [4,61]. There is significant correlation between total tocopherol plasma content and the lipophilic antioxidant capacity [89]. Tocopherols get into plasma via lymphatic chylomicrons [90].

Lycopene and β -carotene are lipid-soluble pigments from the carotenoid group [88]. Both β -carotene and lycopene can quench singlet oxygen without being degraded and react with free radicals such as peroxy, hydroxyl, and superoxide [91–93]. Lycopene plasma levels (0.5–1.0 μM) are slightly higher than β -carotene (0.3–0.6 μM). Lycopene is endowed with a very high antioxidant capacity [61,91] while exhibiting no provitamin A activity, in contrast with β -carotene [94]. The estimated half-life of lycopene elimination from human plasma is approximately 2–3 days [95], but can increase to 10-fold with carotenoid-depleted diet [96]. Plasma levels of β -carotene show conflicting data, reflecting the complexities involving its absorption, interactions, and metabolism [88].

Ubiquinol-10, the reduced form of coenzyme Q₁₀, is a free radical scavenger able to regenerate α -tocopherol, to prevent peroxidative damage to cell membranes, and to minimize oxidative burden by dehydrogenation to ubiquinone-10 (the oxidized form of coenzyme Q₁₀) [97,98]. Ubiquinol has been shown to be an important antioxidant in plasma lipoproteins, promoting inhibition of LDL oxidation with an even better efficiency than lycopene, β -carotene, or α -tocopherol [99]. In human plasma, the concentration of ubiquinol is 0.4–1.0 μM [4,61] and the ratio of Ubiquinol:Ubiquinone has therefore been proposed as an oxidative stress marker [100].

The plasma thiol-proteome

The plasma proteome has more than 3000 components, with a small number of proteins exhibiting very high abundance and an increasingly known group of proteins at lower abundance. In human plasma, protein thiol concentration is in the 400–600 μM range and they are common targets of oxidative modifications [24,27,101]. HSA itself represents more than half of the protein mass, and together with the other most abundant proteins comprises >95% of the total protein mass [102]. Thiol oxidoreductases, which are present at high levels intracellularly and perform important functions, are found in different concentrations and conditions in plasma.

HSA can act as a powerful extracellular antioxidant [61,101] due to its bound ligands and because HSA constitutes an important target for oxidants, with its Cys³⁴ being critical for this property. HSA mixed disulphide pool is essentially equivalent to that of protein-mixed disulphides, since 70–90% of protein-bound thiols are linked to HSA and the binding to other proteins is relatively minor [102]. HSA plasma pool is found as a mixture of mercaptalbumin, bearing the reduced thiol, corresponding to 75% of total HSA in healthy adults, and non-mercaptalbumin, bearing the modified thiol. The latter consists mainly of mixed disulphides of HSA with LMW thiols [4]. A fraction of HSA thiols is nitrosylated, with concentrations <0.2 μM , being able to act as a nitric oxide reservoir and to undergo transnitrosation reactions [103,104].

Besides HSA, only GPx3 was identified amongst the 150 most abundant antioxidant polypeptides in plasma proteome [102], although the other selenoprotein, Trx reductase (TrxR), is also present. Together with other selenoproteins, they represent the majority of extracellular selenium circulating in plasma [105] and show antioxidant activity. Plasma GPx represents approximately 20% of total selenium [106,107] and GPx3 is the main ROS-detoxifying selenoenzyme in human plasma, catalyzing the reduction of extracellular H₂O₂ and lipid hydroperoxides at the expense of reducing equivalents from Trx as well as glutathione [108,109]. Plasma GPx is composed of four 22-kDa subunits, with the incorporated selenium present as selenocysteine and a glycosylated N-terminal region [110,111]. GPx3 concentration was reported as 0.5–0.8 μM (approximately 3.8–43.8 $\mu\text{g/ml}$) in healthy adults [112].

Trx and TrxR compose an important antioxidant oxidoreductase system involved in a number of clinical conditions [113]. Variable Trx plasma levels have been found in several diseases, such as AIDS [114], rheumatoid arthritis [115,116], acute lung injury [117], acute myocardial infarction [118], chronic heart failure [119], sepsis [120], and also in patients undergoing cardiac surgery with cardiopulmonary bypass [121]. Plasma TrxR activity and protein were

also detected in healthy individuals, the latter at a median concentration of $3 \times 10^{-4} \mu\text{M}$ (approximately 18 ng/ml) [122]. Recently, a C-terminal truncated form of Trx called Trx-80 having proinflammatory cytokine-like activities was shown to exhibit an age-dependent increase in human plasma [123]. Another Trx superfamily member, glutaredoxin (Grx), present in mammalian cells in at least two types (Grx 1 and 2) was also measured in human plasma. Total Grx concentration was reported as $456 \pm 284 \text{ ng/ml}$ in patients undergoing cardiac surgery [121], while in healthy donors Grx1 concentration was $13.4 \pm 7.9 \text{ ng/ml}$ and Grx2 was not detected [124].

Quiescinsulphhydryl oxidase (QSOX) can introduce disulphide bonds into reduced proteins, with the accompanying reduction of oxygen to H_2O_2 . QSOX is found intracellularly, but specially as cell-surface and secreted forms. QSOX1 is present at approximately 25 nM in normal adult human serum; however, circulating QSOX1 is unlikely to significantly contribute to the oxidation of monothiols in blood [125]. Of note, QSOX1 is present at high concentrations in FBS and markedly decreases after birth, suggesting possible developmental roles [126]. Some studies suggested the potential for QSOX to be a diagnostic marker for diseases such as pancreatic cancer [127] and heart failure [128]. Other Trx superfamily oxidoreductases include those from the PDI family. PDIA1, as well as protein disulphide isomerase A3 (Erp57) and protein disulphide isomerase A6 (Erp5), are involved in platelet activation and thrombosis [129]. PDIA1 is essential for thrombus formation *in vivo* [130,131], since neither platelet accumulation nor fibrin generation occur in the injured vascular bed in the absence of PDIA1 secretion. In parallel, PDIs have become an important antithrombotic target. However, the presence and implications of circulating PDIs are unclear. PDIA1 is secreted by cultured endothelial cells basally in the absence of cell lysis [132]. Furthermore, it has been proposed that the absence of PDIA1 and probably of other PDIs from plasma in the intact circulatory system is a mechanism to suppress thrombus formation and maintain vascular patency in the absence of vascular injury [133]. PDIA1 secretion under acute shear stress in endothelial cells is able to oxidize $\alpha 5$ -integrin [131], although secreted PDIA1 most often acts as a thiol reductase [134]. PDIA1 interaction with Nox NADPH oxidases may provide an additional redox connection [135].

EC-SOD or SOD3 is a major antioxidant enzyme found in plasma as well as lymph and synovial fluid [136–138]. EC-SOD occurs in several tissues; however, higher amounts are present in vascular tissue, mainly in vascular smooth muscle cells [139]. While the basal circulating concentration of EC-SOD is relatively low ($<0.2 \mu\text{g/ml}$), soluble plasma EC-SOD equilibrates with an immobilized form bound to extracellular matrix ligands such as heparan sulphate at the endothelial cell surface [140]. In fact, EC-SOD is a homotetrameric protein with a heparin-binding domain which can modulate EC-SOD plasma concentration; intravenous heparin infusion displaces EC-SOD from endothelial cell surface into plasma, leading to increased EC-SOD activity [141].

S-nitrosothiols in human plasma

Nitric oxide (NO) has a wide variety of functions including several roles in protein modification and oxidation of thiols, resulting in S-nitrosothiols (SNO). Plasma SNOs have been detected *in vivo* as LMW nitrosothiols (S-nitrosoglutathione, S-nitrosocysteine) and high-molecular weight nitrosothiols (S-nitrosoalbumin). Concentrations of the former are lower than those of the latter [103,142]. Initial measurements of SNO in plasma of healthy individuals reported values of approximately $7 \mu\text{M}$, while free NO levels were nearly $3 \mu\text{M}$. SNO accounts for approximately 96% of NO covalently bound to proteins, of which approximately 82% is the S-nitrosoalbumin adduct. Nitrosothiols have a much longer physiologic half-life than NO, e.g. the half-life of S-nitrosoalbumin is approximately 40 min compared with a half-life of NO of approximately 3 s [143]. The incubation of plasma with S-nitrosoglutathione and S-nitrosocysteine resulted in their rapid decomposition and $<10\%$ of the initial concentration was recovered after 10–15 min, suggesting that SNO species are in a dynamic state in human plasma. Moreover, virtually all NO was recovered as S-nitrosoalbumin, indicating that HSA is the major determinant of such rapid decomposition. Additionally, the intravenous infusion of NO solution results in the transport and delivery of NO as SNO, which associates with systemic hemodynamic effects such as vasodilation in conduit and resistance vessels [144].

Overall, assessment of plasma levels of specific thiol oxidoreductases, antioxidant enzymes, and of the thiol proteome in general remains highly incipient and has the potential to reveal yet unexplored information on several (patho)physiological aspects of human disease.

Thiol pools and disease states

Several disease states associate with oxidation and modification of thiols in a way to suggest their causal role in corresponding pathologic events. In parallel, such thiol changes can potentially work as diagnostic or prognostic markers in these conditions, even if being only non-causally related epiphenomena. In fact, the plasma pools of CyS/CySS

Table 2 Plasma thiols in ageing

Condition	Associated findings	Concentration or E_h	Reference
Older individuals (>60 years) with ARMD; younger (<60 years) healthy individuals	↑ plasma GSH in younger compared with older individuals; ↑ plasma and whole-blood GSSG levels (E_h : approximately 45 mV > oxidized) in older compared with young controls	GSH* compared with GSSG ^(†) levels Young: 1.89 compared with 84 Older: 1.32 compared with 111 ARMD: 1.18 compared with 131	[149]
Healthy individuals aged 19–85 years	GSH/GSSG and CyS/CySS pools became oxidized with increased age; Cys oxidation: from 19 to 85 years (0.16 mV/year); GSH oxidation: mainly > 45 years (0.7 mV/year)	E_h † GSH/GSSG: -140 ± 11 CyS/CySS: -72 ± 14	[145]
ARMD patients under antioxidant supplementation	Antioxidants protected against Cys depletion and oxidation of E_h -Cys over time; ↑ plasma oxidation of E_h -Cys (+9 mV with and +12 mV without antioxidant supplementation)	E_h Cys ^(*,‡) No antioxidant: -72.16 to -67.32 Antioxidant: -71.50 to -74.04	[151]
Healthy individuals aged 21–92 years	GSH and Cys-Gly levels showed strong inverse correlation with age; ↑ oxidized Cys and Hcy levels in elderly individuals	Values individually reported	[27]
Healthy individuals aged 20–93 years	Age-dependent ↓ in protein thiol and ↑ in cysteinylated and homocysteinylated plasma protein levels	Total levels † Cys: 237.11 ± 36.61 Glutathione: 5.79 ± 1.80 Hcy: 11.25 ± 4.44 Cys-Gly: 19.74 ± 3.93	[217]

All concentration values were reported in micromolar, except as specified. Abbreviation: ARMD, age-related macular degeneration.

*reported as mean values.

†reported as mean \pm S.D.

‡mean change from initial (1.7 years after randomization) to last (6.7 years after randomization) evaluation.
whole blood levels

and GSH/GSSG vary in association with different conditions [145–147]. A paradigmatic example is ageing, as discussed below, in which the values for their redox potentials become more positive (oxidized) in association with age [146,147]. GSH/GSSG plasma ratios are reportedly unchanged until approximately 45 years of age, and then become oxidized at a rate of 0.7 mV/year over the entire age range from 18 to 93 years [146]. In addition, a more oxidizing thiol/disulphide redox state is associated with multiple other circumstances such as chronic alcohol abuse [148], anticancer therapy [149], type 2 diabetes [150], and cigarette smoking [151]. Here, we briefly discuss available information on thiol redox markers in ageing and selected diseases. Rather than enumerating the extensive and sometimes controversial literature on a large number of diseases, we concentrate on technically reliable information related to CVDs and atherosclerosis, cancer, and neurodegenerative diseases, since these ones have more available data and allow one to model possible patterns potentially applicable to most disorders. Along this line, in the subsequent section we discuss in more detail one specific disease, namely atherosclerosis, for which there is more substantial information.

Age-dependent plasma oxidation has been proposed by several studies in healthy individuals and patients with age-related diseases. These studies are discriminated with specific details in Table 2. Such investigations have pointed to a consistent behavior of increased oxidation in plasma E_h and particularly Cys oxidation in older individuals, with higher GSH pool in young ones [149]. Accordingly, the steady-state level of CyS/CySS is increasingly more oxidized than GSH/GSSG with age. Glutathione oxidation occurs mainly after the age of 45, while Cys has a linear oxidation along the entire ageing range [145]. Other evidences suggest that antioxidant supplementation can protect against depletion of Cys and oxidation of E_h Cys over time [152]. Taken together, these findings demonstrate that oxidative burden increases with age, and in parallel the plasma compartment has its redox components affected. Besides, it supports the interpretation that plasma redox values are active indicators of the systemic balance between oxidant/antioxidant processes and redox signaling along life. In parallel, a conclusion from these studies is that every investigation in which plasma thiol redox state is somehow assessed has to take the effects of ageing very closely.

There are several evidences that CVDs are associated with the redox state of thiol/disulphide systems. Such studies are discussed in detail in Table 3 and further discussion on atherosclerosis is detailed in the next section. Elevated Cys plasma levels associate with increased risk for atherosclerosis and coronary heart disease (CHD) [153,154]. However, interpretation of these findings is confounded by the fact that they refer to the total amount of Cys and there was no separation of reduced and oxidized forms. The few studies addressing CySS levels in the cardiovascular context suggest that CySS is a reliable independent predictor of vascular disease and associates with endothelial dysfunction [155] as well as risk of death in coronary artery disease patients [17]. Increasing Hcy plasma levels are known to associate with risk of CVD as well. In contrast, GSH levels showed an inverse association with risk [17,156]. In summary, these findings suggest that plasma redox processes accompany the natural history of CVD development and

Table 3 Plasma thiols in CVDs and atherosclerosis

Condition	Associated findings	Concentration or E_h	Reference
Early-onset peripheral atherosclerosis	↑ all Hcy and Cys forms (except CyS) in patients compared with controls; ↑ CyS and CyS/total Cys ratio in controls compared with patients	<i>Patients compared with Controls</i> (*,§) tCys: 321.9 compared with 292.9 CyS: 11.4 compared with 14.5 CyS/tCys: 3.6 compared with 5.0 tHcy: 15.7 compared with 12.7	[218]
Hyperlipidemia	Total Cys was a risk factor for atherosclerosis; ↑ Cys levels in patients with CVD compared with asymptomatic subjects	<i>Cys levels</i> (†) Asymptomatic: 239.1 ± 44.3 Symptomatic: 254.7 ± 47.7	[153]
Healthy subjects	Total Cys associated with several cardiovascular risk factors	<i>Total levels</i> (‡) Cys: 268.0	[219]
Vascular disease	↑ CHD risk from low (<250 μM) to high (>275 μM) tCys; combined ↓ tCys and ↑ tHcy yield highest risk for vascular disease ()	<i>Total levels</i> (†) Cys: 275.3 ± 35.3 Hcy: not clearly specified	[152]
CHD	↑ tCys and tHcy in patients compared with controls; such differences were age dependent in patients	<i>Patients compared with Controls</i> (†) Cys: 254.6 ± 8.1 compared with 187.1 ± 7.7 Hcy: 16.3 ± 0.6 compared with 9.2 ± 0.7	[220]
Healthy non-smoking subjects free from CVD	↑ GSH, E_h GSH/GSSG, CySS correlated with increased IMT; E_h GSH/GSSG was an independent predictor of early atherosclerosis	GSH (†): 1.6 ± 0.5 E_h GSH/GSSG (†): -126 ± 7.5 CySS (†): 83 ± 15.9	[221]
Healthy non-smoking volunteers	Correlation between endothelial function and oxidized thiols; CySS and CySSG levels were predictors of flow-mediated vasodilatation	CySS (†): 83.7 ± 15.9 CySSG (†): 2.92 ± 1.09	[155]
Patients undergoing cardiac catheterization	↓ GSH levels were independent predictors of impaired microvascular function (lower CFVR and higher HMR) and necrotic core	GSH (†): 1.2 ± 0.4	[155]
Acute coronary syndrome	Cys and glutathione were associated with the combined end point of AMI, stroke, and/or cardiovascular death	<i>Total levels</i> (†) Cys: 275 Glutathione: 7.8 Hcy: 16.6	[215]
Population with coronary artery disease	↑ CySS, ↓ GSH levels, or ↑ CySS/GSH ratio associate with risk of future death over 5 years	CySS (): 97.6 GSH (): 1.17 GSSG (): 0.02	[17]

All concentration values were reported in micromolar, except as specified. Abbreviations: CFVR, coronary flow velocity reserve; HMR, hyperemic microvascular resistance; IMT, intima-media thickness; t, total values.

* reported as mean values.

† reported as mean ± S.D.

‡ reported as median values.

§ concentration reported for male patients (fasting) – there is >1 value for each aminothiols reported in the present study.

|| CHD, CVD, and Peripheral Vascular Disease.

its associated complications. A more general conclusion is that a systemic alteration in redox homeostasis accompanies CVD. However, with the possible exception of CySS, thiol redox-signaling measurements have not yet shown a clear-cut behavior pattern in such diseases, either because their incomplete or inadequate assessment or because of the complex natural history of such disease processes, with their distinct stages and associated complications.

The role of oxidative processes in cancer is complex, with actions related both to tumor development (mainly during tumorigenesis) and suppression of tumor growth and metastatization (mainly with established tumors) [157]. Thus, it is not surprising that some studies of the association between circulating thiol pools in different types of cancers, detailed in Table 4, are limited and not clearly consistent. Increased total Cys levels are associated with decreased risk for breast, esophageal, and gastric cancer [158,159]. In contrast, in colorectal cancer (CRC) the opposite association is observed [160]. Increased Hcy levels are associated with higher risk for breast [161] and CRC [160]. In other cases, i.e. lung cancer, thiols and disulphides were found to decrease in the same way [162] or there was no clear association between thiols and risk for cancer development, i.e. head/neck and esophagus cancer [163]. Exposure to NAC increases tumor growth, metastasis, and mortality in mice with lung cancer and melanoma [164]. This effect is similar to that of other antioxidants, indicating that the antioxidant protection *per se* is deleterious in such cases. Overall, these data indicate a complex interplay between circulating thiols and risk for cancer so that data have to be analyzed under a strict context-dependence, particularly regarding the type and stage of cancer.

The relationship amongst plasma thiols, severity, and duration of neurodegenerative diseases has been investigated through several studies (Table 5). However, as mentioned before for cancers, the findings do not allow the identification of a robust pattern. In Alzheimer (AD) and Parkinson diseases (PD), Hcy levels are often elevated and

Table 4 Plasma thiols in cancer

Condition	Associated findings	Concentration	Reference
Women with breast cancer	↑ circulating τ Cys levels associated with ↓ breast cancer risk (dose-dependent); incidence ↓ approximately 50% with Cys levels >351 nM	Total Cys levels (^{†,§}) Cases: 301 ± 58 Controls: 305 ± 59	[157]
Women with invasive breast cancer	No difference in Cys or Hcy levels between cancer cases and controls	Cys compared with Hcy levels (^{†,§}) Cases: 204.6 compared with 8.8 Controls: 203.1 compared with 8.4	[160]
Esophageal and gastric cancer	↑ Cys levels associated with ↓ risk in both OSCC and GCA patients	Cys levels (^{†,§,}) OSCC cases: 191 GCA cases: 198	[158]
Women with CRC	↑ Hcy and ↓ Cys associated with increased CRC risk; Hcy levels associated with proximal colon tumors and Cys with rectal tumors	Cases compared with Controls ([†]) Cys: 283.9 ± 1.1 compared with 284.0 ± 1.1 Hcy: 9.42 ± 1.31 compared with 9.12 ± 1.28	[159]
Advanced non-small-cell lung cancer	Thiol/disulphide ratio unchanged; reduced thiol, total thiol, and disulphide levels ↓ in patients compared with controls	Cancer compared with Controls ([†]) τ Thiol: 300.6 ± 68.9 compared with 354.0 ± 79.5 Reduced thiol: 266.1 ± 64.5 compared with 313.0 ± 78.2 Disulphide: 17.2 ± 5.6 compared with 20.2 ± 4.4	[161]
Cancer of head and neck, esophagus, stomach	Cys levels associated with gastric carcinoma risk while Cys-Gly levels showed an inverse association. No association with OSSC and HNSCC risk	Cys compared with Cys-Gly (^{†,§,}) GCA: 310.1 compared with 223.0 OSSC: 308.5 compared with 230.9 HNSCC: 305.1 compared with 221.9	[162]

All concentration values were reported in micromolar, except as specified. Abbreviations: OSCC, oesophageal squamous cell carcinoma; GCA, gastric cardia adenocarcinoma; HNSCC, head and neck squamous cell carcinoma; τ , total values.

* reported as mean values.

† reported as mean ± S.D.

‡ reported as median values.

§ reported as nM concentration.

|| serum levels.

Table 5 Plasma thiols in neurodegenerative diseases

Condition	Associated findings	Concentration	Refs
Idiopathic PD and controls	Hcy but not Cys and Cys-Gly levels were elevated in patients compared with controls; Cys-Gly levels were related to the duration of PD	Patients compared with Controls ([†]) Hcy: 17.5 ± 6.5 compared with 13.2 ± 3.7 Cys: 243.1 ± 33.4 compared with 253.7 ± 38.2 Cys-Gly: 52.3 ± 7.3 compared with 53.3 ± 6.7	[222]
Patients followed for AD incidence	Hcy levels were unrelated to AD and cognitive decline over time	Hcy levels ([†]): 15.6	[223]
HD patients and HD carriers	Both HD patients and HD carriers ↓ GSH levels compared with controls; GSH levels were similar in both HD groups, which also showed ↑ lipid peroxidation	GSH levels ([†]) HD patients: 8.2 Controls: 11.4 HD carriers: 8.5 Controls: 13.8	[167]
AD and controls	↓ GSH levels correlated with degree of cognitive impairment; ↑ Hcy and Cys levels in AD while GSH was unchanged compared with controls	Patients compared with Controls ([†]) Hcy: 12.6 compared with 9.6 Cys: 120.1 compared with 107.2 GSH: 2.7 compared with 2.5	[168]
Subjects without dementia	↑ Hcy levels were a risk factor for dementia and AD	Hcy levels (^{†,‡}) Men: 13.1 ± 6.3 Women: 13.0 ± 7.0	[223]
PD treated with levodopa and healthy controls	Association between ↑ plasma Hcy levels and dementia in PD; ↑ Hcy levels in PD compared with controls; ↑ Hcy levels in PDD compared with PDnD patients	Hcy levels ([†]) PD patients: 17.5 ± 10.2 Controls: 11 ± 4.1 PDD patients: 20.7 ± 12.1 PDnD patients: 15.8 ± 8.5	[166]

All concentration values were reported in micromolar, except as specified. Abbreviations: HD, Huntington disease; PDD, PD with dementia; PDnD, PD without dementia.

* reported as mean ± S.D.

† reported as median values.

‡ values were also reported individually for different age groups.

associated with development of dementia [165,166]. Furthermore, lower levels of glutathione are found in Alzheimer and Huntingtons' patients, although associated with cognitive impairment only in the former [167,168]. In summary, the relevance of plasma thiol pools in neurodegenerative diseases require further study, mainly because it is not clear to what extent plasma thiols reflect alterations within brain tissue.

Effects of plasma thiol pools in disease pathophysiology: the case of atherosclerosis

The discussion in the section *Thiol pools and disease states* indicates that in several cases overall associations between plasma thiol redox pools and disease, but many such data are correlative observations derived from case–control studies. While it is useful to investigate thiol pools as possible disease biomarkers, they tell little about possible causal associations or mechanistic inferences. Here, rather than enumerating the multiple possible roles of plasma thiols in disease pathophysiology, we briefly mention such issues with a focus on one specific disease, namely atherosclerosis. The involvement of redox processes in the natural history of atherosclerosis includes three major groups of events. First, the predominant component likely relates to cell signaling routes involved in proliferation, growth, migration, senescence and apoptosis, well known to associate with substantial rates of oxidant generation, as well as thiol-disulphide exchange reactions [169]. Second, the involvement of inflammatory processes is intrinsically linked to redox (dys)regulation. Third, lipoprotein oxidation and formation of oxidized lipid products is believed to be a major trigger of early atheroma development, as well as of later complications [170,171]. A general framework for the mechanisms whereby extracellular redox processes affect intracellular redox processes and, conversely, how intracellular processes contribute to plasma redox milieu suggests roles for the oxidation of specific proteins [172], as well as for secreted microparticles [172], which are highly thiol redox-modulated [173] and circulating miRNAs [174].

Along these lines, the involvement of plasma thiol redox pools in atherosclerosis can be more directly linked to effects in: (i) lipoprotein oxidation; (ii) inflammatory responses of circulating blood elements; (iii) cell membrane oxidative damage; (iv) thrombosis; (v) modification of plasma and extracellular matrix proteins, and (vi) signaling events in endothelial cells.

It is well accepted that LDL oxidation is a crucial step in atherogenesis, being able to promote lipoprotein uptake by macrophages, foam cell formation, ensuing proinflammatory responses and endothelial dysfunction, amongst many other responses [175]. LDL oxidation is believed to occur predominantly within macrophages, endothelial, and smooth muscle cells [176]. While the extent of plasma LDL oxidation is debatable [169], plasma redox state can directly or indirectly modulate such reaction. Generally, plasma by itself tends to suppress LDL oxidation, although surprisingly little is known about the direct effects of thiol pools in such process. However, thiol donor compounds, as well as several other antioxidants, can decrease LDL oxidation [171,177,178], and the plasma levels of some endogenous antioxidants, such as unconjugated bilirubin, can protect against LDL oxidation [179]. An important caveat in these studies is that the methods to address LDL oxidation *in vitro* may not reflect the corresponding *in vivo* conditions. In fact, *in vitro* studies of total reducing activity of plasma indicate that antioxidants able to scavenge peroxy radicals *in vitro* may not be as efficient *in vivo* [180]. In addition to extracellular antioxidants, intracellular glutathione can be protective against the response to oxidized lipoproteins, since lower glutathione levels in macrophages enhance CD36 expression and oxidized LDL (oxLDL) uptake [181]. In parallel, some components of plasma can enhance LDL oxidation. Hcy can enhance metal-catalyzed LDL oxidation [182] and γ -glutamyl transpeptidase, an enzyme found in serum and membranes, supports LDL oxidation through removal of the cysteine-protective γ -glutamyl glutathione residue [183]. Vascular peroxidase VPO-1 reportedly triggers LDL oxidation [184]. Also, oxidants impair antioxidant effects of albumin against LDL oxidation [185]. Furthermore, oxidized lipids can impair the atheroprotection afforded by high-density lipoprotein (HDL) particles. Plasma cysteinemia supports enhanced plasma levels of Apolipoprotein-A1, a critical HDL component [186]. Lipid hydroperoxides in oxLDL oxidize the cysteine of lecithin-cholesterol acyl transferase, potentially impairing HDL particle maturation [187]. In this context, a main HDL component is the enzyme paraoxonase-1, which drives the hydrolysis of aryl esters including Hcy thiolactone [188]. Altogether, the failure of exogenous antioxidant compounds to protect against atherosclerosis development in humans [189] argues against a monotonic role of oxidized lipids in this process and suggests complexities that also extend to the roles of plasma components in disease evolution.

The roles of plasma components in inflammation closely follow their lipid oxidation effects, but may also occur directly. For example, macrophage glutathione reduction potential, potentially influenced by plasma redox milieu, is a strong predictor of macrophage content in the lesions [190]. Accordingly, increased macrophage glutathione reductase activity reduces the severity of atherosclerosis in mice [180]. Moreover, MPO, known to be highly secreted by inflammatory neutrophils, was shown to oxidize cyanide to cyanate, generating accumulation of carbamylated

substrates in atheromas [191]. In addition, MPO can chlorinate and nitrate Apolipoprotein A-I tyrosines in human atheromas [192]. Additional studies implicate Prdx in atherosclerosis via inflammatory mechanisms. Studies in genetically modified mice indicate that Prdx4, the only known secreted Prdx, contributes to protect against oxidative and proinflammatory changes and atherosclerosis development [193,194]. This is in line with the reported atheroprotective effects of the macrophage Prdx1, which supports lipid autophagy and cholesterol efflux [195] and Prdx2, highly expressed in endothelial and immune cells [196]. Endothelial Prdx1 is also a marker of atheroprotective shear stress [197]. UA levels have been shown to be associated with cardiovascular risk and this association is reported to associate with inflammatory microvascular impairment and endothelial dysfunction [73,198]. Indeed, higher levels of circulating UA were predictive of carotid atherosclerosis [199]. The mechanisms whereby UA promotes atherosclerosis, despite its important antioxidant effect discussed previously, are possibly dependent on the significant proinflammatory effects of UA crystals. Direct proinflammatory roles of UA [75], as well as a prooxidant activity of urate hydroperoxide have also been described [200].

Decreased H₂S production induced by genetic deletion of cystathione – γ -lyase promotes enhanced atherosclerosis development accompanied by impaired plasma redox homeostasis, with increased levels of malondialdehyde and lower levels of GSH and antioxidant thiol proteins such as GPx and glutathione reductase, leading to enhanced subendothelial uptake of oxLDL [201,202]. Moreover, secretion of cystathione- γ -lyase and cystathione β -synthase by endothelial cells and hepatocytes contributes to generate H₂S from circulating Hcy, supporting endothelial protection [203]. The selenoprotein GPx4 has the unique cell membrane-protecting effects through its activity of reducing phospholipid hydroperoxides within membrane lipid bilayers and lipoproteins. α -tocopherol sufficiency is essential to prevent the massive thrombosis associated with GPx4 genetic deletion [204]. Importantly, the major – if not the principal [169] – extracellular antioxidant enzyme GPx3 exerts crucial roles to limit arterial thrombosis and platelet activation. Deficiency of GPx3 in humans or mice models associate with redox-sensitive platelet activation, endothelial dysfunction, and enhanced risk of ischemic stroke in young [169].

Oxidative changes in extracellular matrix compounds can closely parallel the oxidation of CyS/CySS couple that occurs in some conditions. In fact, an oxidized extracellular E_h CyS/CySS E_h was shown to importantly modulate fibroblast proliferation and fibronectin expression in the context of lung fibrosis [205]. Matrix-degrading enzymes such as matrix metalloproteinases (MMP) are crucially involved in atherosclerotic plaque rupture and ulceration involved in superimposed thrombosis and clinical events [206,207]. MMPs are redox-sensitive and may be activated by oxidants, which target the autoinhibitory cysteine that co-ordinates the active site zinc residue. Under sustained inflammation, proMMP is activated by oxidative S-glutathionylation at the autoinhibitory domain via S-nitroglutathione (GSNO₂), a product of the reaction between ONOO⁻ and glutathione [208].

Interplay of endothelial and vessel wall cell thiol pools with those from plasma can potentially affect intracellular redox balance. Endogenous E_h values for GSH/GSSG usually vary between –260 and –200 mV, concentrating around –230 to –220 mV in rest non-dividing cells. More negative (reduced) potential associates with cell proliferation [7], while moderately less negative E_h values associate with cell differentiation and marked less negative E_h (–170 to –150 mV) associate with cell apoptosis [209]. Surprisingly little, however, is known about direct effects of exogenous (plasma) thiol pools on vascular proliferation. We showed previously that intraarterial challenge of GSSG during and immediately after vascular injury triggered a marked increase in the vascular repair reaction, with enhanced neointimal thickening and vessel wall proliferation. The effects of GSSG were prevented by co-infusion of GSH, indicating that they result from an imbalance of thiol-disulphide redox equilibrium [210]. More recently, we showed that perivascularly induced immunoneutralization of extracellular PDIA1 pool induced vessel narrowing due to constrictive remodeling [211]. PDIA1 is highly expressed in human coronary atheromas [212]. This further adds to the significant importance of intra- and extracellular redox processes in vascular remodeling and mechanoadaptive responses [213]. In fact, an anticonstrictive remodeling effect of EC-SOD was shown previously by us [214].

Conclusion and critical analysis: is there such a thing as ‘plasma oxidative stress’?

The concepts discussed along the present review indicate a complex picture for the interpretation of plasma thiol redox, with several individual pools subjected to interplay of multiple influences, sometimes with opposing roles. Although reliable measurements of thiol-disulphide pools in plasma may provide relevant pathophysiological information, as discussed above, their practical utility as individual biomarkers of such processes is a concrete possibility, which remains however unclear. Therefore, the possible usefulness of these measurements as risk factors for diseases

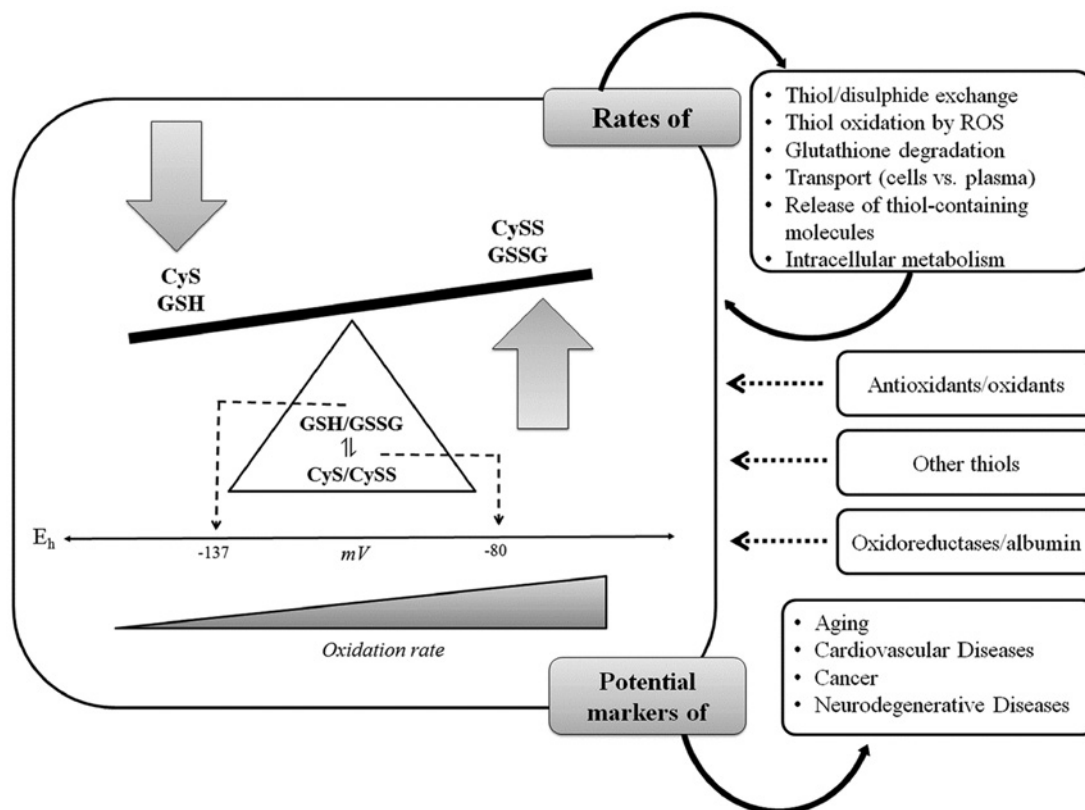


Figure 4. Summary of plasma thiol/disulphide network as potential markers of disease states

The equilibrium of thiol/disulphide pairs can be shaped by ambient redox status and influenced by several components acting as a ‘redox network’. Thiol/disulphide pairs (i.e. glutathione and cysteine) are not in equilibrium in plasma and the E_h of Cys/CySS (–80 mV) pool is more oxidized than that of GSH/GSSG pool (–137 mV). Their steady-state levels are the result of several processes occurring simultaneously, such as the rates of: thiol–disulphide exchange reactions, thiol oxidation by ROS, enzymatic extracellular degradation of glutathione, transport between cells and plasma, release of thiol-containing molecules such as HSA, and intracellular metabolism. Moreover, plasma antioxidants, oxidants, oxidoreductases, albumin, and other thiols influence the equilibrium values of plasma thiol redox pools. These thiol networks are associated with several diseases that involve oxidation and modification of thiols, indicating that they play a critical role in ageing and several pathologic conditions (i.e. cardiovascular and neurodegenerative diseases, cancer) and can potentially work as diagnostic or prognostic markers.

[17,153,215] is a promising application of these concepts. Also, further investigation of specific thiol-proteins (described as ‘professional thiol redox proteins’ in Table 1) remains a potentially important but yet unexplored area. Figure 4 is a summary of these concepts.

In this regard, a critical caveat is needed. Measurements of plasma thiol oxidative burden are frequently regarded as representing ‘plasma oxidative stress’ or even ‘organismal oxidative stress’ resulting from localized or systemic diseases which *per se* associate with oxidant generation. These inferences present significant conceptual and factual limitations. First, the issues discussed in this review indicate that it is naïve to assume that a single measurement reflects the altered thiol balance of plasma. In addition, redox processes in plasma result from events occurring in plasma itself, as well as in large-scale oxidative shifts resulting from endothelial processes in generalized inflammation including atherosclerosis, sepsis and vasculitis, thrombotic diseases such as systemic coagulation disorders and specific hematologic conditions such as malignancies. In addition, as discussed above, ageing associates with a more oxidizing plasma phenotype [146,216]. In parallel, the concept that localized diseases in specific organs such as cancer and even localized atheroma plaques will promote oxidative shifts in plasma thiols is challenging. The model that a few diseased cells would ‘leak’ oxidants into plasma and alter its thiol redox state has little support from elementary quantitative calculations. Rather, it is likely that the reported association between thiol redox shifts and CVD, for example [17,152,214] implicates plasma oxidative shift associated with systemic factors such as endothelial dysfunction due to lipid-related inflammation, diabetes, systemic thrombotic tendency, and other unknown factors. In this

regard, plasma oxidative shifts reflect a risk factor for vascular disease rather than possible roles as biomarkers of the localized disease process itself. Finally, the term ‘organismal oxidative stress’ or systemic oxidative stress is conceptually inappropriate, given the complexity and multiple compartments of whole body organization. Thus, even for ageing, it is not obvious whether plasma thiol redox shifts reflect specific processes occurring in plasma rather than those from other specific organs such as heart, kidneys, and central nervous system.

Bearing these caveats in mind, the discussion highlighted in this review indicates that further study of plasma thiol pools at the mechanistic level has a concrete potential to bring relevant information regarding the behavior of compartmentalized oxidative burden in normal and disease tissues. This, in turn, may provide information regarding risk for specific diseases, as well as disease staging and prognosis. Overall, it is likely that assessment of individual thiol pools and the yet unexplored range of thiol-proteins specific to any given (patho)physiological condition have a more solid and logical perspective to yield novel relevant information than the general assessment of thiol redox state.

Funding

This work was supported by the FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) [Cepid Redoxoma grant number 2013/07937-8 (to F.R.M.L.)]; Scholarship grant [number 2014/20595-1 (to P.V.S.O.)].

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Author contribution

P.V.S.O. designed and wrote the review and F.R.M.L. supervised the work and wrote the review.

Abbreviations

3NT, 3-nitro-tyrosine; AA, ascorbic acid; CHD, coronary heart disease; CRC, colorectal cancer; CVD, cardiovascular disease; Cys, reduced cysteine; CySS, cystine (the disulphide form of cysteine); Cys-Gly, cysteinylglycine; EC-SOD, extracellular superoxide dismutase; E_h , redox potential; GPx, glutathione peroxidase; Grx, glutaredoxin; GSSG, disulphide-oxidized glutathione; HSA, human serum albumin; Hcy, homocysteine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LMW, low molecular weight; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NAC, *N*-acetylcysteine; oxLDL, oxidized LDL; PDI, protein disulphide isomerase; Prdx, peroxiredoxin; QSOX, quiescin sulphydryl oxidase; ROS, reactive oxygen species; SNO, S-nitrosothiol; Trx, thioredoxin; TrxR, Trx reductase; UA, uric acid; VPO1, vascular peroxidase 1.

References

- Eaton, P. (2006) Protein thiol oxidation in health and disease: techniques for measuring disulfides and related modifications in complex protein mixtures. *Free Radic. Biol. Med.* **40**, 1889–1899, <https://doi.org/10.1016/j.freeradbiomed.2005.12.037>
- Chen, K., Thomas, S.R. and Keaney, Jr, J.F. (2003) Beyond LDL oxidation: ROS in vascular signal transduction. *Free Radic. Biol. Med.* **35**, 117–132, [https://doi.org/10.1016/S0891-5849\(03\)00239-9](https://doi.org/10.1016/S0891-5849(03)00239-9)
- Biteau, B., Labarre, J. and Toledano, M.B. (2003) ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* **425**, 980–984, <https://doi.org/10.1038/nature02075>
- Turell, L., Radi, R. and Alvarez, B. (2013) The thiol pool in human plasma: the central contribution of albumin to redox processes. *Free Radic. Biol. Med.* **65**, 244–253, <https://doi.org/10.1016/j.freeradbiomed.2013.05.050>
- Yi, M.C., Melkonian, A.V., Ousey, J.A. and Khosla, C. (2018) Endoplasmic reticulum-resident protein 57 (ERp57) oxidatively inactivates human transglutaminase 2. *J. Biol. Chem.* **293**, 2640–2649, <https://doi.org/10.1074/jbc.RA117.001382>
- Jensen, K.S., Hansen, R.E. and Winther, J.R. (2009) Kinetic and thermodynamic aspects of cellular thiol-disulfide redox regulation. *Antioxid. Redox Signal.* **11**, 1047–1058, <https://doi.org/10.1089/ars.2008.2297>
- Kemp, M., Go, Y.M. and Jones, D.P. (2008) Nonequilibrium thermodynamics of thiol/disulfide redox systems: a perspective on redox systems biology. *Free Radic. Biol. Med.* **44**, 921–937, <https://doi.org/10.1016/j.freeradbiomed.2007.11.008>
- Buettner, G.R. (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* **300**, 535–543, <https://doi.org/10.1006/abbi.1993.1074>
- Jones, D.P. and Liang, Y. (2009) Measuring the poise of thiol/disulfide couples *in vivo*. *Free Radic. Biol. Med.* **47**, 1329–1338, <https://doi.org/10.1016/j.freeradbiomed.2009.08.021>
- Go, Y.M. and Jones, D.P. (2013) Thiol/disulfide redox states in signaling and sensing. *Crit. Rev. Biochem. Mol. Biol.* **48**, 173–181, <https://doi.org/10.3109/10409238.2013.764840>
- Singhal, S.S., Yadav, S., Roth, C. and Singhal, J. (2009) RLIP76: a novel glutathione-conjugate and multi-drug transporter. *Biochem. Pharmacol.* **77**, 761–769, <https://doi.org/10.1016/j.bcp.2008.10.006>

- 12 Awasthi, S., Singhal, S.S., Sharma, R., Zimniak, P. and Awasthi, Y.C. (2003) Transport of glutathione conjugates and chemotherapeutic drugs by RLP76 (RALBP1): a novel link between G-protein and tyrosine kinase signaling and drug resistance. *Int. J. Cancer* **106**, 635–646, <https://doi.org/10.1002/ijc.11260>
- 13 Gainey, D., Short, S. and McCoy, K.L. (1996) Intracellular location of cysteine transport activity correlates with productive processing of antigen disulfide. *J. Cell. Physiol.* **168**, 248–254, [https://doi.org/10.1002/\(SICI\)1097-4652\(199608\)168:2%3c248::AID-JCP3%3e3.0.CO;2-P](https://doi.org/10.1002/(SICI)1097-4652(199608)168:2%3c248::AID-JCP3%3e3.0.CO;2-P)
- 14 Watts, S.D., Torres-Salazar, D., Divito, C.B. and Amara, S.G. (2014) Cysteine transport through excitatory amino acid transporter 3 (EAAT3). *PLoS ONE* **9**, e109245, <https://doi.org/10.1371/journal.pone.0109245>
- 15 Ito, K. and Inaba, K. (2008) The disulfide bond formation (Dsb) system. *Curr. Opin. Struct. Biol.* **18**, 450–458, <https://doi.org/10.1016/j.sbi.2008.02.002>
- 16 Jones, D.P. (2006) Extracellular redox state: refining the definition of oxidative stress in aging. *Rejuvenation Res.* **9**, 169–181, <https://doi.org/10.1089/rej.2006.9.169>
- 17 Patel, R.S., Ghasemzadeh, N., Eapen, D.J., Sher, S., Arshad, S., Ko, Y.A. et al. (2016) Novel biomarker of oxidative stress is associated with risk of death in patients with coronary artery disease. *Circulation* **133**, 361–369, <https://doi.org/10.1161/CIRCULATIONAHA.115.019790>
- 18 Yuan, L. and Kaplowitz, N. (2009) Glutathione in liver diseases and hepatotoxicity. *Mol. Aspects Med.* **30**, 29–41, <https://doi.org/10.1016/j.mam.2008.08.003>
- 19 Hansen, R.E., Roth, D. and Winther, J.R. (2009) Quantifying the global cellular thiol-disulfide status. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 422–427, <https://doi.org/10.1073/pnas.0812149106>
- 20 Jones, D.P. and Go, Y.M. (2010) Redox compartmentalization and cellular stress. *Diabetes Obes. Metab.* **12**, 116–125, <https://doi.org/10.1111/j.1463-1326.2010.01266.x>
- 21 Jones, D.P., Go, Y.M., Anderson, C.L., Ziegler, T.R., Kinkade, Jr, J.M. and Kirlin, W.G. (2004) Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. *FASEB J.* **18**, 1246–1248, <https://doi.org/10.1096/fj.03-0971fje>
- 22 Ookhtens, M. and Kaplowitz, N. (1998) Role of the liver in interorgan homeostasis of glutathione and cyst(e)ine. *Semin. Liver Dis.* **18**, 313–329, <https://doi.org/10.1055/s-2007-1007167>
- 23 Moriarty-Craige, S.E. and Jones, D.P. (2004) Extracellular thiols and thiol/disulfide redox in metabolism. *Annu. Rev. Nutr.* **24**, 481–509, <https://doi.org/10.1146/annurev.nutr.24.012003.132208>
- 24 Mansoor, M.A., Svardal, A.M. and Ueland, P.M. (1992) Determination of the *in vivo* redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. *Anal. Biochem.* **200**, 218–229, [https://doi.org/10.1016/0003-2697\(92\)90456-H](https://doi.org/10.1016/0003-2697(92)90456-H)
- 25 Nolin, T.D., McMennamin, M.E. and Himmelfarb, J. (2007) Simultaneous determination of total homocysteine, cysteine, cysteinylglycine, and glutathione in human plasma by high-performance liquid chromatography: application to studies of oxidative stress. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **852**, 554–561, <https://doi.org/10.1016/j.jchromb.2007.02.024>
- 26 Andersson, A., Isaksson, A., Brattstrom, L. and Hultberg, B. (1993) Homocysteine and other thiols determined in plasma by HPLC and thiol-specific postcolumn derivatization. *Clin. Chem.* **39**, 1590–1597
- 27 Giustarini, D., Dalle-Donne, I., Lorenzini, S., Milzani, A. and Rossi, R. (2006) Age-related influence on thiol, disulfide, and protein-mixed disulfide levels in human plasma. *J. Gerontol. A Biol. Sci. Med. Sci.* **61**, 1030–1038, <https://doi.org/10.1093/gerona/61.10.1030>
- 28 Jones, D.P., Carlson, J.L., Mody, V.C., Cai, J., Lynn, M.J. and Sternberg, P. (2000) Redox state of glutathione in human plasma. *Free Radic. Biol. Med.* **28**, 625–635, [https://doi.org/10.1016/S0891-5849\(99\)00275-0](https://doi.org/10.1016/S0891-5849(99)00275-0)
- 29 Ueland, P.M. (1995) Homocysteine species as components of plasma redox thiol status. *Clin. Chem.* **41**, 340–342
- 30 Aebi, S., Assereto, R. and Lauterburg, B.H. (1991) High-dose intravenous glutathione in man. Pharmacokinetics and effects on cyst(e)ine in plasma and urine. *Eur. J. Clin. Invest.* **21**, 103–110, <https://doi.org/10.1111/j.1365-2362.1991.tb01366.x>
- 31 Hankey, G.J. and Eikelboom, J.W. (1999) Homocysteine and vascular disease. *Lancet* **354**, 407–413, [https://doi.org/10.1016/S0140-6736\(98\)11058-9](https://doi.org/10.1016/S0140-6736(98)11058-9)
- 32 Ganguly, P. and Alam, S.F. (2015) Role of homocysteine in the development of cardiovascular disease. *Nutr. J.* **14**, 6, <https://doi.org/10.1186/1475-2891-14-6>
- 33 Carballal, S., Radi, R., Kirk, M.C., Barnes, S., Freeman, B.A. and Alvarez, B. (2003) Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. *Biochemistry* **42**, 9906–9914, <https://doi.org/10.1021/bi027434m>
- 34 Kleinman, W.A. and Richie, Jr, J.P. (2000) Status of glutathione and other thiols and disulfides in human plasma. *Biochem. Pharmacol.* **60**, 19–29, [https://doi.org/10.1016/S0006-2952\(00\)00293-8](https://doi.org/10.1016/S0006-2952(00)00293-8)
- 35 Chen, Y.H., Yao, W.Z., Geng, B., Ding, Y.L., Lu, M., Zhao, M.W. et al. (2005) Endogenous hydrogen sulfide in patients with COPD. *Chest* **128**, 3205–3211, <https://doi.org/10.1378/chest.128.5.3205>
- 36 Zhao, W., Zhang, J., Lu, Y. and Wang, R. (2001) The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. *EMBO J.* **20**, 6008–6016, <https://doi.org/10.1093/emboj/20.21.6008>
- 37 Han, Y., Qin, J., Chang, X., Yang, Z. and Du, J. (2006) Hydrogen sulfide and carbon monoxide are in synergy with each other in the pathogenesis of recurrent febrile seizures. *Cell. Mol. Neurobiol.* **26**, 101–107, <https://doi.org/10.1007/s10571-006-8848-z>
- 38 Cheng, Y., Wu, D., Yang, G., Li, X., Huang, D., Geng, B. et al. (2008) The role of hydrogen sulfide system in the pathogenesis of renovascular hypertension in rats. *J. Geriatric Cardiol.* **5**, 45–49
- 39 Furne, J., Saeed, A. and Levitt, M.D. (2008) Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R1479–R1485, <https://doi.org/10.1152/ajpregu.90566.2008>
- 40 Deneke, S.M. (2000) Thiol-based antioxidants. *Curr. Top Cell Regul.* **36**, 151–180, [https://doi.org/10.1016/S0070-2137\(01\)80007-8](https://doi.org/10.1016/S0070-2137(01)80007-8)
- 41 Jones, D.P. (2008) Radical-free biology of oxidative stress. *Am. J. Physiol. Cell Physiol.* **295**, C849–C868, <https://doi.org/10.1152/ajpcell.00283.2008>

- 42 Andersson, A., Lindgren, A. and Hultberg, B. (1995) Effect of thiol oxidation and thiol export from erythrocytes on determination of redox status of homocysteine and other thiols in plasma from healthy subjects and patients with cerebral infarction. *Clin. Chem.* **41**, 361–366
- 43 Pendyala, L., Schwartz, G., Smith, P., Zdanowicz, J., Murphy, M. and Hausheer, F. (2003) Modulation of plasma thiols and mixed disulfides by BNP7787 in patients receiving paclitaxel/cisplatin therapy. *Cancer Chemother. Pharmacol.* **51**, 376–384
- 44 Mansoor, M.A., Guttormsen, A.B., Fiskerstrand, T., Refsum, H., Ueland, P.M. and Svardal, A.M. (1993) Redox status and protein binding of plasma amino thiols during the transient hyperhomocysteinemia that follows homocysteine administration. *Clin. Chem.* **39**, 980–985
- 45 Hildebrandt, W., Sauer, R., Bonaterra, G., Dugi, K.A., Edler, L. and Kinscherf, R. (2015) Oral N-acetylcysteine reduces plasma homocysteine concentrations regardless of lipid or smoking status. *Am. J. Clin. Nutr.* **102**, 1014–1024, <https://doi.org/10.3945/ajcn.114.101964>
- 46 Atkuri, K.R., Mantovani, J.J., Herzenberg, L.A. and Herzenberg, L.A. (2007) N-Acetylcysteine—a safe antidote for cysteine/glutathione deficiency. *Curr. Opin. Pharmacol.* **7**, 355–359, <https://doi.org/10.1016/j.coph.2007.04.005>
- 47 Ezerina, D., Takano, Y., Hanaoka, K., Urano, Y. and Dick, T.P. (2018) N-acetyl cysteine functions as a fast-acting antioxidant by triggering intracellular H₂S and sulfane sulfur production. *Cell Chem. Biol.* **25**, 447.e4–459.e4, <https://doi.org/10.1016/j.chembiol.2018.01.011>
- 48 Forman, H.J., Bernardo, A. and Davies, K.J. (2016) What is the concentration of hydrogen peroxide in blood and plasma? *Arch. Biochem. Biophys.* **603**, 48–53, <https://doi.org/10.1016/j.abb.2016.05.005>
- 49 Pastori, D., Pignatelli, P., Carnevale, R. and Violi, F. (2015) Nox-2 up-regulation and platelet activation: novel insights. *Prostaglandins Other Lipid Mediat.* **120**, 50–55, <https://doi.org/10.1016/j.prostaglandins.2015.03.010>
- 50 White, C.R., Darley-Usmar, V., Berrington, W.R., McAdams, M., Gore, J.Z., Thompson, J.A. et al. (1996) Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8745–8749, <https://doi.org/10.1073/pnas.93.16.8745>
- 51 Lymar, S.V. and Hurst, K. (1995) Rapid reaction between peroxynitrite ion and carbon dioxide: implications for biological activity. *J. Am. Chem. Soc.* **117**, 8867–8868, <https://doi.org/10.1021/ja00139a027>
- 52 Torres-Rasgado, E., Fouret, G., Carbonneau, M.A. and Leger, C.L. (2007) Peroxynitrite mild nitration of albumin and LDL-albumin complex naturally present in plasma and tyrosine nitration rate-albumin impairs LDL nitration. *Free Radic. Res.* **41**, 367–375, <https://doi.org/10.1080/10715760601064706>
- 53 Augusto, O., Bonini, M.G., Amanso, A.M., Linares, E., Santos, C.C. and De Menezes, S.L. (2002) Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. *Free Radic. Biol. Med.* **32**, 841–859, [https://doi.org/10.1016/S0891-5849\(02\)00786-4](https://doi.org/10.1016/S0891-5849(02)00786-4)
- 54 White, C.R., Brock, T.A., Chang, L.Y., Crapo, J., Briscoe, P., Ku, D. et al. (1994) Superoxide and peroxynitrite in atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1044–1048, <https://doi.org/10.1073/pnas.91.3.1044>
- 55 Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V. and Moncada, S. (1993) Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett.* **330**, 181–185, [https://doi.org/10.1016/0014-5793\(93\)80269-Z](https://doi.org/10.1016/0014-5793(93)80269-Z)
- 56 Pullar, J.M., Vissers, M.C. and Winterbourn, C.C. (2000) Living with a killer: the effects of hypochlorous acid on mammalian cells. *IUBMB Life* **50**, 259–266, <https://doi.org/10.1080/15216540051080958>
- 57 Cheng, G., Li, H., Cao, Z., Qiu, X., McCormick, S., Thannickal, V.J. et al. (2011) Vascular peroxidase-1 is rapidly secreted, circulates in plasma, and supports dityrosine cross-linking reactions. *Free Radic. Biol. Med.* **51**, 1445–1453, <https://doi.org/10.1016/j.freeradbiomed.2011.07.002>
- 58 Ma, Q.L., Zhang, G.G. and Peng, J. (2013) Vascular peroxidase 1: a novel enzyme in promoting oxidative stress in cardiovascular system. *Trends Cardiovasc. Med.* **23**, 179–183, <https://doi.org/10.1016/j.tcm.2012.11.002>
- 59 Bhave, G., Cummings, C.F., Vanacore, R.M., Kumagai-Cresse, C., Ero-Tolliver, I.A., Rafi, M. et al. (2012) Peroxidase forms sulfilimine chemical bonds using hypohalous acids in tissue genesis. *Nat. Chem. Biol.* **8**, 784–790, <https://doi.org/10.1038/nchembio.1038>
- 60 Denicola, A., Souza, J.M. and Radi, R. (1998) Diffusion of peroxynitrite across erythrocyte membranes. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3566–3571, <https://doi.org/10.1073/pnas.95.7.3566>
- 61 Motchnik, P.A., Frei, B. and Ames, B.N. (1994) Measurement of antioxidants in human blood plasma. *Methods Enzymol.* **234**, 269–279, [https://doi.org/10.1016/0076-6879\(94\)34094-3](https://doi.org/10.1016/0076-6879(94)34094-3)
- 62 Stocker, R. (2016) Antioxidant defenses in human blood plasma and extra-cellular fluids. *Arch. Biochem. Biophys.* **595**, 136–139, <https://doi.org/10.1016/j.abb.2015.11.021>
- 63 Winkler, B.S., Orselli, S.M. and Rex, T.S. (1994) The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. *Free Radic. Biol. Med.* **17**, 333–349, [https://doi.org/10.1016/0891-5849\(94\)90019-1](https://doi.org/10.1016/0891-5849(94)90019-1)
- 64 Wang, Y., Yang, M., Lee, S.G., Davis, C.G., Kenny, A., Koo, S.I. et al. (2012) Plasma total antioxidant capacity is associated with dietary intake and plasma level of antioxidants in postmenopausal women. *J. Nutr. Biochem.* **23**, 1725–1731, <https://doi.org/10.1016/j.jnutbio.2011.12.004>
- 65 Frei, B., Stocker, R. and Ames, B.N. (1988) Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9748–9752, <https://doi.org/10.1073/pnas.85.24.9748>
- 66 Peluffo, G., Calcerrada, P., Piacenza, L., Pizzano, N. and Radi, R. (2009) Superoxide-mediated inactivation of nitric oxide and peroxynitrite formation by tobacco smoke in vascular endothelium: studies in cultured cells and smokers. *Am. J. Physiol. Heart Circ. Physiol.* **296**, H1781–H1792, <https://doi.org/10.1152/ajpheart.00930.2008>
- 67 Sies, H., Stahl, W. and Sundquist, A.R. (1992) Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. *Ann. N.Y. Acad. Sci.* **669**, 7–20, <https://doi.org/10.1111/j.1749-6632.1992.tb17085.x>
- 68 Nordberg, J. and Arner, E.S. (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.* **31**, 1287–1312, [https://doi.org/10.1016/S0891-5849\(01\)00724-9](https://doi.org/10.1016/S0891-5849(01)00724-9)
- 69 May, J.M. (1999) Is ascorbic acid an antioxidant for the plasma membrane? *FASEB J.* **13**, 995–1006, <https://doi.org/10.1096/fasebj.13.9.995>
- 70 Frei, B., Stocker, R., England, L. and Ames, B.N. (1990) Ascorbate: the most effective antioxidant in human blood plasma. *Adv. Exp. Med. Biol.* **264**, 155–163, https://doi.org/10.1007/978-1-4684-5730-8_24

- 71 Ames, B.N., Cathcart, R., Schwiers, E. and Hochstein, P. (1981) Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6858–6862, <https://doi.org/10.1073/pnas.78.11.6858>
- 72 Lotito, S.B. and Frei, B. (2004) The increase in human plasma antioxidant capacity after apple consumption is due to the metabolic effect of fructose on urate, not apple-derived antioxidant flavonoids. *Free Radic. Biol. Med.* **37**, 251–258, <https://doi.org/10.1016/j.freeradbiomed.2004.04.019>
- 73 Feig, D.I., Kang, D.H. and Johnson, R.J. (2008) Uric acid and cardiovascular risk. *N. Engl. J. Med.* **359**, 1811–1821, <https://doi.org/10.1056/NEJMra0800885>
- 74 Martinon, F., Petrilili, V., Mayor, A., Tardivel, A. and Tschopp, J. (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237–241, <https://doi.org/10.1038/nature04516>
- 75 Braga, T.T., Forni, M.F., Correa-Costa, M., Ramos, R.N., Barbuto, J.A., Branco, P. et al. (2017) Soluble Uric Acid Activates the NLRP3 Inflammasome. *Sci. Rep.* **7**, 39884, <https://doi.org/10.1038/srep39884>
- 76 Skinner, K.A., White, C.R., Patel, R., Tan, S., Barnes, S., Kirk, M. et al. (1998) Nitrosation of uric acid by peroxynitrite. Formation of a vasoactive nitric oxide donor. *J. Biol. Chem.* **273**, 24491–24497, <https://doi.org/10.1074/jbc.273.38.24491>
- 77 Tomaro, M.L. and Battle, A.M. (2002) Bilirubin: its role in cytoprotection against oxidative stress. *Int. J. Biochem. Cell Biol.* **34**, 216–220, [https://doi.org/10.1016/S1357-2725\(01\)00130-3](https://doi.org/10.1016/S1357-2725(01)00130-3)
- 78 Dohi, K., Satoh, K., Ohtaki, H., Shioda, S., Miyake, Y., Shindo, M. et al. (2005) Elevated plasma levels of bilirubin in patients with neurotrauma reflect its pathophysiological role in free radical scavenging. *In Vivo* **19**, 855–860
- 79 Shekeeb Shahab, M., Kumar, P., Sharma, N., Narang, A. and Prasad, R. (2008) Evaluation of oxidant and antioxidant status in term neonates: a plausible protective role of bilirubin. *Mol. Cell. Biochem.* **317**, 51–59, <https://doi.org/10.1007/s11010-008-9807-4>
- 80 Troughton, J.A., Woodside, J.V., Young, I.S., Arveiler, D., Amouyel, P., Ferrieres, J. et al. (2007) Bilirubin and coronary heart disease risk in the Prospective Epidemiological Study of Myocardial Infarction (PRIME). *Eur. J. Cardiovasc. Prev. Rehabil.* **14**, 79–84, <https://doi.org/10.1097/01.hjr.0000230097.81202.9f>
- 81 Perlstein, T.S., Pande, R.L., Beckman, J.A. and Creager, M.A. (2008) Serum total bilirubin level and prevalent lower-extremity peripheral arterial disease: National Health and Nutrition Examination Survey (NHANES) 1999 to 2004. *Arterioscler. Thromb. Vasc. Biol.* **28**, 166–172, <https://doi.org/10.1161/ATVBAHA.107.153262>
- 82 Chan, K.H., O'Connell, R.L., Sullivan, D.R., Hoffmann, L.S., Rajamani, K., Whiting, M. et al. (2013) Plasma total bilirubin levels predict amputation events in type 2 diabetes mellitus: the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study. *Diabetologia* **56**, 724–736, <https://doi.org/10.1007/s00125-012-2818-4>
- 83 Clark, J.E., Foresti, R., Sarathchandra, P., Kaur, H., Green, C.J. and Motterlini, R. (2000) Heme oxygenase-1-derived bilirubin ameliorates postschismic myocardial dysfunction. *Am. J. Physiol. Heart Circ. Physiol.* **278**, H643–H651, <https://doi.org/10.1152/ajpheart.2000.278.2.H643>
- 84 Dore, S. and Snyder, S.H. (1999) Neuroprotective action of bilirubin against oxidative stress in primary hippocampal cultures. *Ann. N.Y. Acad. Sci.* **890**, 167–172, <https://doi.org/10.1111/j.1749-6632.1999.tb07991.x>
- 85 Kato, Y., Shimazu, M., Kondo, M., Uchida, K., Kumamoto, Y., Wakabayashi, G. et al. (2003) Bilirubin rinse: A simple protectant against the rat liver graft injury mimicking heme oxygenase-1 preconditioning. *Hepatology* **38**, 364–373, <https://doi.org/10.1053/jhep.2003.50300>
- 86 Burton, G.W. and Ingold, K.U. (1981) Autoxidation of biological molecules. 1. Antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *J. Am. Chem. Soc.* **103**, 6472–6477, <https://doi.org/10.1021/ja00411a035>
- 87 Schneider, C. (2005) Chemistry and biology of vitamin E. *Mol. Nutr. Food Res.* **49**, 7–30, <https://doi.org/10.1002/mnfr.200400049>
- 88 Schwedhelm, E., Maas, R., Troost, R. and Boger, R.H. (2003) Clinical pharmacokinetics of antioxidants and their impact on systemic oxidative stress. *Clin. Pharmacokinet.* **42**, 437–459, <https://doi.org/10.2165/00003088-200342050-00003>
- 89 Muller, L., Theile, K. and Bohm, V. (2010) *In vitro* antioxidant activity of tocopherols and tocotrienols and comparison of vitamin E concentration and lipophilic antioxidant capacity in human plasma. *Mol. Nutr. Food Res.* **54**, 731–742, <https://doi.org/10.1002/mnfr.200900399>
- 90 Traber, M.G., Burton, G.W., Hughes, L., Ingold, K.U., Hidaka, H., Malloy, M. et al. (1992) Discrimination between forms of vitamin E by humans with and without genetic abnormalities of lipoprotein metabolism. *J. Lipid Res.* **33**, 1171–1182
- 91 Di Mascio, P., Kaiser, S. and Sies, H. (1989) Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch. Biochem. Biophys.* **274**, 532–538, [https://doi.org/10.1016/0003-9861\(89\)90467-0](https://doi.org/10.1016/0003-9861(89)90467-0)
- 92 Schierle, J., Bretzel, W., Bühler, I., Faccin, N., Hess, D., Steiner, K. et al. (1997) Content and isomeric ratio of lycopene in food and human blood plasma. *Food Chem.* **59**, [https://doi.org/10.1016/S0308-8146\(96\)00177-X](https://doi.org/10.1016/S0308-8146(96)00177-X)
- 93 Conn, P.F., Schalch, W. and Truscott, T.G. (1991) The singlet oxygen and carotenoid interaction. *J. Photochem. Photobiol. B* **11**, 41–47, [https://doi.org/10.1016/1011-1344\(91\)80266-K](https://doi.org/10.1016/1011-1344(91)80266-K)
- 94 Sies, H. and Stahl, W. (1998) Lycopene: antioxidant and biological effects and its bioavailability in the human. *Proc. Soc. Exp. Biol. Med.* **218**, 121–124, <https://doi.org/10.3181/00379727-218-44285a>
- 95 Stahl, W. and Sies, H. (1996) Lycopene: a biologically important carotenoid for humans? *Arch. Biochem. Biophys.* **336**, 1–9, <https://doi.org/10.1006/abbi.1996.0525>
- 96 Burri, B.J., Neidlinger, T.R. and Clifford, A.J. (2001) Serum carotenoid depletion follows first-order kinetics in healthy adult women fed naturally low carotenoid diets. *J. Nutr.* **131**, 2096–2100, <https://doi.org/10.1093/jn/131.8.2096>
- 97 Tang, P.H., Miles, M.V., DeGrauw, A., Hershey, A. and Pesce, A. (2001) HPLC analysis of reduced and oxidized coenzyme Q(10) in human plasma. *Clin. Chem.* **47**, 256–265
- 98 Franke, A.A., Morrison, C.M., Bakke, J.L., Custer, L.J., Li, X. and Cooney, R.V. (2010) Coenzyme Q10 in human blood: native levels and determinants of oxidation during processing and storage. *Free Radic. Biol. Med.* **48**, 1610–1617, <https://doi.org/10.1016/j.freeradbiomed.2010.03.002>
- 99 Stocker, R., Bowry, V.W. and Frei, B. (1991) Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does alpha-tocopherol. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1646–1650, <https://doi.org/10.1073/pnas.88.5.1646>

- 100 Weber, C., Jakobsen, T.S., Mortensen, S.A., Paulsen, G. and Holmer, G. (1994) Effect of dietary coenzyme Q10 as an antioxidant in human plasma. *Mol. Aspects Med.* **15**, s97–s102, [https://doi.org/10.1016/0098-2997\(94\)90018-3](https://doi.org/10.1016/0098-2997(94)90018-3)
- 101 Hu, M.L. (1994) Measurement of protein thiol groups and glutathione in plasma. *Methods Enzymol.* **233**, 380–385, [https://doi.org/10.1016/S0076-6879\(94\)33044-1](https://doi.org/10.1016/S0076-6879(94)33044-1)
- 102 Hortin, G.L., Sviridov, D. and Anderson, N.L. (2008) High-abundance polypeptides of the human plasma proteome comprising the top 4 logs of polypeptide abundance. *Clin. Chem.* **54**, 1608–1616, <https://doi.org/10.1373/clinchem.2008.108175>
- 103 Rassaf, T., Feelisch, M. and Kelm, M. (2004) Circulating NO pool: assessment of nitrite and nitroso species in blood and tissues. *Free Radic. Biol. Med.* **36**, 413–422, <https://doi.org/10.1016/j.freeradbiomed.2003.11.011>
- 104 Tsikas, D. and Frolich, J.C. (2002) S-nitrosoalbumin plasma levels in health and disease: facts or artifacts? Value of analytical chemistry in nitric oxide clinical research. *Circ. Res.* **90**, e39
- 105 Burk, R.F. and Hill, K.E. (2009) Selenoprotein P-expression, functions, and roles in mammals. *Biochim. Biophys. Acta* **1790**, 1441–1447, <https://doi.org/10.1016/j.bbagen.2009.03.026>
- 106 Deagen, J.T., Butler, J.A., Zachara, B.A. and Whanger, P.D. (1993) Determination of the distribution of selenium between glutathione peroxidase, selenoprotein P, and albumin in plasma. *Anal. Biochem.* **208**, 176–181, <https://doi.org/10.1006/abio.1993.1025>
- 107 Reyes, L.H., Marchante-Gayón, J.M., Garcia Alonso, J.I. and Sanz-Medel, A. (2003) Quantitative speciation of selenium in human serum by affinity chromatography coupled to post-column isotope dilution analysis ICP-MS. *J. Anal. Atom. Spectrometry* **18**, 1210–1216, <https://doi.org/10.1039/B305455A>
- 108 Bjornstedt, M., Xue, J., Huang, W., Akesson, B. and Holmgren, A. (1994) The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. *J. Biol. Chem.* **269**, 29382–29384
- 109 Saito, Y., Hayashi, T., Tanaka, A., Watanabe, Y., Suzuki, M., Saito, E. et al. (1999) Selenoprotein P in human plasma as an extracellular phospholipid hydroperoxide glutathione peroxidase. Isolation and enzymatic characterization of human selenoprotein p. *J. Biol. Chem.* **274**, 2866–2871, <https://doi.org/10.1074/jbc.274.5.2866>
- 110 Takahashi, K., Avissar, N., Whitin, J. and Cohen, H. (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. *Arch. Biochem. Biophys.* **256**, 677–686, [https://doi.org/10.1016/0003-9861\(87\)90624-2](https://doi.org/10.1016/0003-9861(87)90624-2)
- 111 Maddipati, K.R. and Marnett, L.J. (1987) Characterization of the major hydroperoxide-reducing activity of human plasma. Purification and properties of a selenium-dependent glutathione peroxidase. *J. Biol. Chem.* **262**, 17398–17403
- 112 Jacobson, G.A., Narkowicz, C., Tong, Y.C. and Peterson, G.M. (2006) Plasma glutathione peroxidase by ELISA and relationship to selenium level. *Clin. Chim. Acta* **369**, 100–103, <https://doi.org/10.1016/j.cca.2006.01.007>
- 113 Becker, K., Gromer, S., Schirmer, R.H. and Muller, S. (2000) Thioredoxin reductase as a pathophysiological factor and drug target. *Eur. J. Biochem.* **267**, 6118–6125, <https://doi.org/10.1046/j.1432-1327.2000.01703.x>
- 114 Nakamura, H., De Rosa, S.C., Yodoi, J., Holmgren, A., Ghezzi, P., Herzenberg, L.A. et al. (2001) Chronic elevation of plasma thioredoxin: inhibition of chemotaxis and curtailment of life expectancy in AIDS. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2688–2693, <https://doi.org/10.1073/pnas.041624998>
- 115 Maurice, M.M., Nakamura, H., Gringhuis, S., Okamoto, T., Yoshida, S., Kullmann, F. et al. (1999) Expression of the thioredoxin-thioredoxin reductase system in the inflamed joints of patients with rheumatoid arthritis. *Arthritis Rheum.* **42**, 2430–2439, [https://doi.org/10.1002/1529-0131\(199911\)42:11%3c2430::AID-ANR22%3e3.0.CO;2-6](https://doi.org/10.1002/1529-0131(199911)42:11%3c2430::AID-ANR22%3e3.0.CO;2-6)
- 116 Jikimoto, T., Nishikubo, Y., Koshiba, M., Kanagawa, S., Morinobu, S., Morinobu, A. et al. (2002) Thioredoxin as a biomarker for oxidative stress in patients with rheumatoid arthritis. *Mol. Immunol.* **38**, 765–772, [https://doi.org/10.1016/S0161-5890\(01\)00113-4](https://doi.org/10.1016/S0161-5890(01)00113-4)
- 117 Callister, M.E., Burke-Gaffney, A., Quinlan, G.J., Nicholson, A.G., Florio, R., Nakamura, H. et al. (2006) Extracellular thioredoxin levels are increased in patients with acute lung injury. *Thorax* **61**, 521–527, <https://doi.org/10.1136/thx.2005.053041>
- 118 Miyamoto, S., Sakamoto, T., Soejima, H., Shimomura, H., Kajiwara, I., Kojima, S. et al. (2003) Plasma thioredoxin levels and platelet aggregability in patients with acute myocardial infarction. *Am. Heart J.* **146**, 465–471, [https://doi.org/10.1016/S0002-8703\(03\)00311-9](https://doi.org/10.1016/S0002-8703(03)00311-9)
- 119 Jekell, A., Hossain, A., Alehagen, U., Dahlstrom, U. and Rosen, A. (2004) Elevated circulating levels of thioredoxin and stress in chronic heart failure. *Eur. J. Heart Fail.* **6**, 883–890, <https://doi.org/10.1016/j.ejheart.2004.03.003>
- 120 Leaver, S.K., MacCallum, N.S., Pingle, V., Hacking, M.B., Quinlan, G.J., Evans, T.W. et al. (2010) Increased plasma thioredoxin levels in patients with sepsis: positive association with macrophage migration inhibitory factor. *Intensive Care Med.* **36**, 336–341, <https://doi.org/10.1007/s00134-009-1640-z>
- 121 Nakamura, H., Vaage, J., Valen, G., Padilla, C.A., Bjornstedt, M. and Holmgren, A. (1998) Measurements of plasma glutaredoxin and thioredoxin in healthy volunteers and during open-heart surgery. *Free Radic. Biol. Med.* **24**, 1176–1186, [https://doi.org/10.1016/S0891-5849\(97\)00429-2](https://doi.org/10.1016/S0891-5849(97)00429-2)
- 122 Soderberg, A., Sahaf, B. and Rosen, A. (2000) Thioredoxin reductase, a redox-active selenoprotein, is secreted by normal and neoplastic cells: presence in human plasma. *Cancer Res.* **60**, 2281–2289
- 123 Couchie, D., Vaisman, B., Abderrazak, A., Mahmood, D.F.D., Hamza, M.M., Canesi, F. et al. (2017) Human plasma thioredoxin-80 increases with age and in ApoE(-/-) mice induces inflammation, angiogenesis, and atherosclerosis. *Circulation* **136**, 464–475, <https://doi.org/10.1161/CIRCULATIONAHA.117.027612>
- 124 Lundberg, M., Fernandes, A.P., Kumar, S. and Holmgren, A. (2004) Cellular and plasma levels of human glutaredoxin 1 and 2 detected by sensitive ELISA systems. *Biochem. Biophys. Res. Commun.* **319**, 801–809, <https://doi.org/10.1016/j.bbrc.2004.04.199>
- 125 Israel, B.A., Jiang, L., Gannon, S.A. and Thorpe, C. (2014) Disulfide bond generation in mammalian blood serum: detection and purification of quiescin-sulfhydryl oxidase. *Free Radic. Biol. Med.* **69**, 129–135, <https://doi.org/10.1016/j.freeradbiomed.2014.01.020>
- 126 Zanata, S.M., Luvizon, A.C., Batista, D.F., Ikegami, C.M., Pedrosa, F.O., Souza, E.M. et al. (2005) High levels of active quiescin Q6 sulfhydryl oxidase (QSOX) are selectively present in fetal serum. *Redox Rep.* **10**, 319–323, <https://doi.org/10.1179/135100005X83699>

- 127 Antwi, K., Hostetter, G., Demeure, M.J., Katchman, B.A., Decker, G.A., Ruiz, Y. et al. (2009) Analysis of the plasma peptidome from pancreas cancer patients connects a peptide in plasma to overexpression of the parent protein in tumors. *J. Proteome Res.* **8**, 4722–4731, <https://doi.org/10.1021/pr900414f>
- 128 Mebazaa, A., Vanpoucke, G., Thomas, G., Verleysen, K., Cohen-Solal, A., Vanderheyden, M. et al. (2012) Unbiased plasma proteomics for novel diagnostic biomarkers in cardiovascular disease: identification of quiescin Q6 as a candidate biomarker of acutely decompensated heart failure. *Eur. Heart J.* **33**, 2317–2324, <https://doi.org/10.1093/eurheartj/ehs162>
- 129 Flaumenhaft, R. and Furie, B. (2016) Vascular thiol isomerases. *Blood* **128**, 893–901, <https://doi.org/10.1182/blood-2016-04-636456>
- 130 Cho, J., Furie, B.C., Coughlin, S.R. and Furie, B. (2008) A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. *J. Clin. Invest.* **118**, 1123–1131
- 131 Reinhardt, C., von Bruhl, M.L., Manukyan, D., Grahl, L., Lorenz, M., Altmann, B. et al. (2008) Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. *J. Clin. Invest.* **118**, 1110–1122
- 132 Araujo, T.L., Zeidler, J.D., Oliveira, P.V., Dias, M.H., Armelin, H.A. and Laurindo, F.R. (2017) Protein disulfide isomerase externalization in endothelial cells follows classical and unconventional routes. *Free Radic. Biol. Med.* **103**, 199–208, <https://doi.org/10.1016/j.freeradbiomed.2016.12.021>
- 133 Bowley, S.R., Fang, C., Merrill-Skoloff, G., Furie, B.C. and Furie, B. (2017) Protein disulfide isomerase secretion following vascular injury initiates a regulatory pathway for thrombus formation. *Nat. Commun.* **8**, 14151, <https://doi.org/10.1038/ncomms14151>
- 134 Soares Moretti, A.I. and Martins Laurindo, F.R. (2017) Protein disulfide isomerases: Redox connections in and out of the endoplasmic reticulum. *Arch. Biochem. Biophys.* **617**, 106–119, <https://doi.org/10.1016/j.abb.2016.11.007>
- 135 Laurindo, F.R., Pescatore, L.A. and Fernandes Dde, C. (2012) Protein disulfide isomerase in redox cell signaling and homeostasis. *Free Radic. Biol. Med.* **52**, 1954–1969, <https://doi.org/10.1016/j.freeradbiomed.2012.02.037>
- 136 Marklund, S.L. (1982) Human copper-containing superoxide dismutase of high molecular weight. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7634–7638, <https://doi.org/10.1073/pnas.79.24.7634>
- 137 Marklund, S.L., Bjelle, A. and Elmqvist, L.G. (1986) Superoxide dismutase isoenzymes of the synovial fluid in rheumatoid arthritis and in reactive arthritides. *Ann. Rheum. Dis.* **45**, 847–851, <https://doi.org/10.1136/ard.45.10.847>
- 138 Nozik-Grayck, E., Suliman, H.B. and Piantadosi, C.A. (2005) Extracellular superoxide dismutase. *Int. J. Biochem. Cell Biol.* **37**, 2466–2471, <https://doi.org/10.1016/j.biocel.2005.06.012>
- 139 Ottaviano, F.G., Handy, D.E. and Loscalzo, J. (2008) Redox regulation in the extracellular environment. *Circ. J.* **72**, 1–16, <https://doi.org/10.1253/circj.72.1>
- 140 Petersen, S.V., Thogersen, I.B., Valnickova, Z., Nielsen, M.S., Petersen, J.S., Poulsen, E.T. et al. (2010) The concentration of extracellular superoxide dismutase in plasma is maintained by LRP-mediated endocytosis. *Free Radic. Biol. Med.* **49**, 894–899, <https://doi.org/10.1016/j.freeradbiomed.2010.06.019>
- 141 Karlsson, K. and Marklund, S.L. (1988) Extracellular superoxide dismutase in the vascular system of mammals. *Biochem. J.* **255**, 223–228
- 142 Goldman, R.K., Vlessis, A.A. and Trunkey, D.D. (1998) Nitrosothiol quantification in human plasma. *Anal. Biochem.* **259**, 98–103, <https://doi.org/10.1006/abio.1998.2651>
- 143 Stamler, J.S., Jaraki, O., Osborne, J., Simon, D.I., Keaney, J., Vita, J. et al. (1992) Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7674–7677, <https://doi.org/10.1073/pnas.89.16.7674>
- 144 Jourdeheil, D., Hallen, K., Feelisch, M. and Grisham, M.B. (2000) Dynamic state of S-nitrosothiols in human plasma and whole blood. *Free Radic. Biol. Med.* **28**, 409–417, [https://doi.org/10.1016/S0891-5849\(99\)00257-9](https://doi.org/10.1016/S0891-5849(99)00257-9)
- 145 Blanco, R.A., Ziegler, T.R., Carlson, B.A., Cheng, P.Y., Park, Y., Cotsonis, G.A. et al. (2007) Diurnal variation in glutathione and cysteine redox states in human plasma. *Am. J. Clin. Nutr.* **86**, 1016–1023, <https://doi.org/10.1093/ajcn/86.4.1016>
- 146 Jones, D.P., Mody, Jr, V.C., Carlson, J.L., Lynn, M.J. and Sternberg, Jr, P. (2002) Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses. *Free Radic. Biol. Med.* **33**, 1290–1300, [https://doi.org/10.1016/S0891-5849\(02\)01040-7](https://doi.org/10.1016/S0891-5849(02)01040-7)
- 147 Hildebrandt, W., Kinscherf, R., Hauer, K., Holm, E. and Droge, W. (2002) Plasma cystine concentration and redox state in aging and physical exercise. *Mech. Ageing Dev.* **123**, 1269–1281, [https://doi.org/10.1016/S0047-6374\(02\)00013-1](https://doi.org/10.1016/S0047-6374(02)00013-1)
- 148 Yeh, M.Y., Burnham, E.L., Moss, M. and Brown, L.A. (2007) Chronic alcoholism alters systemic and pulmonary glutathione redox status. *Am. J. Respir. Crit. Care Med.* **176**, 270–276, <https://doi.org/10.1164/rccm.200611-17220C>
- 149 Jonas, C.R., Puckett, A.B., Jones, D.P., Griffith, D.P., Szeszycki, E.E., Bergman, G.F. et al. (2000) Plasma antioxidant status after high-dose chemotherapy: a randomized trial of parenteral nutrition in bone marrow transplantation patients. *Am. J. Clin. Nutr.* **72**, 181–189, <https://doi.org/10.1093/ajcn/72.1.181>
- 150 Samiec, P.S., Drews-Botsch, C., Flagg, E.W., Kurtz, J.C., Sternberg, Jr, P., Reed, R.L. et al. (1998) Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes. *Free Radic. Biol. Med.* **24**, 699–704, [https://doi.org/10.1016/S0891-5849\(97\)00286-4](https://doi.org/10.1016/S0891-5849(97)00286-4)
- 151 Moriarty, S.E., Shah, J.H., Lynn, M., Jiang, S., Openo, K., Jones, D.P. et al. (2003) Oxidation of glutathione and cysteine in human plasma associated with smoking. *Free Radic. Biol. Med.* **35**, 1582–1588, <https://doi.org/10.1016/j.freeradbiomed.2003.09.006>
- 152 Moriarty-Craige, S.E., Adkison, J., Lynn, M., Gensler, G., Bressler, S., Jones, D.P. et al. (2005) Antioxidant supplements prevent oxidation of cysteine/cystine redox in patients with age-related macular degeneration. *Am. J. Ophthalmol.* **140**, 1020–1026, <https://doi.org/10.1016/j.ajo.2005.06.043>
- 153 El-Khairi, L., Ueland, P.M., Refsum, H., Graham, I.M. and Vollset, S.E. (2001) Plasma total cysteine as a risk factor for vascular disease: The European Concerted Action Project. *Circulation* **103**, 2544–2549, <https://doi.org/10.1161/01.CIR.103.21.2544>
- 154 Jacob, N., Bruckert, E., Giral, P., Foglietti, M.J. and Turpin, G. (1999) Cysteine is a cardiovascular risk factor in hyperlipidemic patients. *Atherosclerosis* **146**, 53–59, [https://doi.org/10.1016/S0021-9150\(99\)00128-8](https://doi.org/10.1016/S0021-9150(99)00128-8)

- 155 Ashfaq, S., Abramson, J.L., Jones, D.P., Rhodes, S.D., Weintraub, W.S., Hooper, W.C. et al. (2008) Endothelial function and aminothiols biomarkers of oxidative stress in healthy adults. *Hypertension* **52**, 80–85, <https://doi.org/10.1161/HYPERTENSIONAHA.107.097386>
- 156 Dhawan, S.S., Eshtehardi, P., McDaniel, M.C., Fike, L.V., Jones, D.P., Quyyumi, A.A. et al. (2011) The role of plasma aminothiols in the prediction of coronary microvascular dysfunction and plaque vulnerability. *Atherosclerosis* **219**, 266–272, <https://doi.org/10.1016/j.atherosclerosis.2011.05.020>
- 157 Liou, G.Y. and Storz, P. (2010) Reactive oxygen species in cancer. *Free Radic. Res.* **44**, 479–496, <https://doi.org/10.3109/10715761003667554>
- 158 Zhang, S.M., Willett, W.C., Selhub, J., Manson, J.E., Colditz, G.A. and Hankinson, S.E. (2003) A prospective study of plasma total cysteine and risk of breast cancer. *Cancer Epidemiol. Biomarkers Prev.* **12**, 1188–1193
- 159 Murphy, G., Fan, J.H., Mark, S.D., Dawsey, S.M., Selhub, J., Wang, J. et al. (2011) Prospective study of serum cysteine levels and oesophageal and gastric cancers in China. *Gut* **60**, 618–623, <https://doi.org/10.1136/gut.2010.225854>
- 160 Miller, J.W., Beresford, S.A., Neuhauser, M.L., Cheng, T.Y., Song, X., Brown, E.C. et al. (2013) Homocysteine, cysteine, and risk of incident colorectal cancer in the Women's Health Initiative observational cohort. *Am. J. Clin. Nutr.* **97**, 827–834, <https://doi.org/10.3945/ajcn.112.049932>
- 161 Lin, J., Lee, I.M., Song, Y., Cook, N.R., Selhub, J., Manson, J.E. et al. (2010) Plasma homocysteine and cysteine and risk of breast cancer in women. *Cancer Res.* **70**, 2397–2405, <https://doi.org/10.1158/0008-5472.CAN-09-3648>
- 162 Dirican, N., Dirican, A., Sen, O., Aynali, A., Atalay, S., Bircan, H.A. et al. (2016) Thiol/disulfide homeostasis: A prognostic biomarker for patients with advanced non-small cell lung cancer? *Redox Rep.* **21**, 197–203, <https://doi.org/10.1179/1351000215Y.0000000027>
- 163 Miranti, E.H., Freedman, N.D., Weinstein, S.J., Abnet, C.C., Selhub, J., Murphy, G. et al. (2016) Prospective study of serum cysteine and cysteinylglycine and cancer of the head and neck, esophagus, and stomach in a cohort of male smokers. *Am. J. Clin. Nutr.* **104**, 686–693, <https://doi.org/10.3945/ajcn.115.125799>
- 164 Sayin, V.I., Ibrahim, M.X., Larsson, E., Nilsson, J.A., Lindahl, P. and Bergo, M.O. (2014) Antioxidants accelerate lung cancer progression in mice. *Sci. Transl. Med.* **6**, 221ra15, <https://doi.org/10.1126/scitranslmed.3007653>
- 165 Seshadri, S., Beiser, A., Selhub, J., Jacques, P.F., Rosenberg, I.H., D'Agostino, R.B. et al. (2002) Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N. Engl. J. Med.* **346**, 476–483, <https://doi.org/10.1056/NEJMoa011613>
- 166 Zoccolella, S., dell'Aquila, C., Abruzzese, G., Antonini, A., Bonuccelli, U., Canesi, M. et al. (2009) Hyperhomocysteinemia in levodopa-treated patients with Parkinson's disease dementia. *Mov. Disord.* **24**, 1028–1033, <https://doi.org/10.1002/mds.22511>
- 167 Klepac, N., Relja, M., Klepac, R., Hecimovic, S., Babic, T. and Trkulja, V. (2007) Oxidative stress parameters in plasma of Huntington's disease patients, asymptomatic Huntington's disease gene carriers and healthy subjects: a cross-sectional study. *J. Neurol.* **254**, 1676–1683, <https://doi.org/10.1007/s00415-007-0611-y>
- 168 McCaddon, A., Hudson, P., Hill, D., Barber, J., Lloyd, A., Davies, G. et al. (2003) Alzheimer's disease and total plasma aminothiols. *Biol. Psychiatry* **53**, 254–260, [https://doi.org/10.1016/S0006-3223\(02\)01451-8](https://doi.org/10.1016/S0006-3223(02)01451-8)
- 169 Loscalzo, J. (2014) Redox dysregulation in vascular pathobiology. *Free Radic. Biol. Med.* **75** (Suppl. 1), S2, <https://doi.org/10.1016/j.freeradbiomed.2014.10.597>
- 170 Navab, M., Anantharamaiah, G.M., Reddy, S.T., Van Lenten, B.J., Ansell, B.J., Fonarow, G.C. et al. (2004) The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. *J. Lipid Res.* **45**, 993–1007, <https://doi.org/10.1194/jlr.R400001-JLR200>
- 171 Mitra, S., Deshmukh, A., Sachdeva, R., Lu, J. and Mehta, J.L. (2011) Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy. *Am. J. Med. Sci.* **342**, 135–142, <https://doi.org/10.1097/MAJ.0b013e318224a147>
- 172 Griffiths, H.R., Dias, I.H., Willetts, R.S. and Devitt, A. (2014) Redox regulation of protein damage in plasma. *Redox Biol.* **2**, 430–435, <https://doi.org/10.1016/j.redox.2014.01.010>
- 173 Benedikter, B.J., Weseler, A.R., Wouters, E.F.M., Savelkoul, P.H.M., Rohde, G.G.U. and Stassen, F.R.M. (2018) Redox-dependent thiol modifications: implications for the release of extracellular vesicles. *Cell. Mol. Life Sci.*, <https://doi.org/10.1007/s00018-018-2806-z>
- 174 Zampetaki, A., Dudek, K. and Mayr, M. (2013) Oxidative stress in atherosclerosis: the role of microRNAs in arterial remodeling. *Free Radic. Biol. Med.* **64**, 69–77, <https://doi.org/10.1016/j.freeradbiomed.2013.06.025>
- 175 Leonarduzzi, G., Gamba, P., Gargiulo, S., Biasi, F. and Poli, G. (2012) Inflammation-related gene expression by lipid oxidation-derived products in the progression of atherosclerosis. *Free Radic. Biol. Med.* **52**, 19–34, <https://doi.org/10.1016/j.freeradbiomed.2011.09.031>
- 176 Quinn, M.T., Parthasarathy, S., Fong, L.G. and Steinberg, D. (1987) Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2995–2998, <https://doi.org/10.1073/pnas.84.9.2995>
- 177 Siow, R.C., Sato, H., Leake, D.S., Pearson, J.D., Bannai, S. and Mann, G.E. (1998) Vitamin C protects human arterial smooth muscle cells against atherogenic lipoproteins: effects of antioxidant vitamins C and E on oxidized LDL-induced adaptive increases in cysteine transport and glutathione. *Arterioscler. Thromb. Vasc. Biol.* **18**, 1662–1670, <https://doi.org/10.1161/01.ATV.18.10.1662>
- 178 Konovalova, G.G., Lisina, M.O., Tikhaze, A.K. and Lankin, V.Z. (2003) A complex of antioxidant vitamins effectively inhibits free-radical oxidation of LDL phospholipids in blood plasma and membrane structures of the liver and myocardium. *Bull. Exp. Biol. Med.* **135**, 143–146, <https://doi.org/10.1023/A:1023863629947>
- 179 Boon, A.C., Hawkins, C.L., Bisht, K., Coombes, J.S., Bakrania, B., Wagner, K.H. et al. (2012) Reduced circulating oxidized LDL is associated with hypocholesterolemia and enhanced thiol status in Gilbert syndrome. *Free Radic. Biol. Med.* **52**, 2120–2127, <https://doi.org/10.1016/j.freeradbiomed.2012.03.002>
- 180 Nguyen-Khoa, T., Massy, Z.A., Witko-Sarsat, V., Thevenin, M., Touam, M., Lambrey, G. et al. (1999) Critical evaluation of plasma and LDL oxidant-trapping potential in hemodialysis patients. *Kidney Int.* **56**, 747–753, <https://doi.org/10.1046/j.1523-1755.1999.00565.x>
- 181 Qiao, M., Kisgati, M., Cholewa, J.M., Zhu, W., Smart, E.J., Sulistio, M.S. et al. (2007) Increased expression of glutathione reductase in macrophages decreases atherosclerotic lesion formation in low-density lipoprotein receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **27**, 1375–1382, <https://doi.org/10.1161/ATVBAHA.107.142109>

- 182 Pfanzagl, B., Tribi, F., Koller, E. and Moslinger, T. (2003) Homocysteine strongly enhances metal-catalyzed LDL oxidation in the presence of cystine and cysteine. *Atherosclerosis* **168**, 39–48, [https://doi.org/10.1016/S0021-9150\(03\)00057-1](https://doi.org/10.1016/S0021-9150(03)00057-1)
- 183 Paolicchi, A., Minotti, G., Tonarelli, P., Tongiani, R., De Cesare, D., Mezzetti, A. et al. (1999) Gamma-glutamyl transpeptidase-dependent iron reduction and LDL oxidation—a potential mechanism in atherosclerosis. *J. Investig. Med.* **47**, 151–160
- 184 Yang, Y., Shi, R., Cao, Z., Zhang, G. and Cheng, G. (2016) VPO1 mediates oxidation of LDL and formation of foam cells. *Oncotarget* **7**, 35500–35511
- 185 Bourdon, E., Loreau, N. and Blache, D. (1999) Glucose and free radicals impair the antioxidant properties of serum albumin. *FASEB J.* **13**, 233–244, <https://doi.org/10.1096/fasebj.13.2.233>
- 186 Nuno-Ayala, M., Guillen, N., Navarro, M.A., Lou-Bonafonte, J.M., Arnal, C., Gascon, S. et al. (2010) Cysteinemia, rather than homocysteinemia, is associated with plasma apolipoprotein A-I levels in hyperhomocysteinemia: lipid metabolism in cystathionine beta-synthase deficiency. *Atherosclerosis* **212**, 268–273, <https://doi.org/10.1016/j.atherosclerosis.2010.04.028>
- 187 Bielicki, J.K. and Forte, T.M. (1999) Evidence that lipid hydroperoxides inhibit plasma lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* **40**, 948–954
- 188 Moya, C. and Manez, S. (2018) Paraoxonases: metabolic role and pharmacological projection. *Naunyn Schmiedebergs Arch. Pharmacol.* **391**, 349–359, <https://doi.org/10.1007/s00210-018-1473-9>
- 189 Niki, E. (2018) Oxidant-specific biomarkers of oxidative stress. Association with atherosclerosis and implication for antioxidant effects. *Free Radic. Biol. Med.* **120**, 425–440, <https://doi.org/10.1016/j.freeradbiomed.2018.04.001>
- 190 Qiao, M., Zhao, Q., Lee, C.F., Tannock, L.R., Smart, E.J., LeBaron, R.G. et al. (2009) Thiol oxidative stress induced by metabolic disorders amplifies macrophage chemotactic responses and accelerates atherogenesis and kidney injury in LDL receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **29**, 1779–1786, <https://doi.org/10.1161/ATVBAHA.109.191759>
- 191 Delporte, C., Zouaoui Boudjeltia, K., Furtmuller, P.G., Maki, R.A., Dieu, M., Noyon, C. et al. (2018) Myeloperoxidase-catalyzed oxidation of cyanide to cyanate: A potential carbamylation route involved in the formation of atherosclerotic plaques? *J. Biol. Chem.* **293**, 6374–6386, <https://doi.org/10.1074/jbc.M117.801076>
- 192 Shao, B., Pennathur, S. and Heinecke, J.W. (2012) Myeloperoxidase targets apolipoprotein A-I, the major high density lipoprotein protein, for site-specific oxidation in human atherosclerotic lesions. *J. Biol. Chem.* **287**, 6375–6386, <https://doi.org/10.1074/jbc.M111.337345>
- 193 Yamada, S. and Guo, X. (2018) Peroxiredoxin 4 (PRDX4): Its critical *in vivo* roles in animal models of metabolic syndrome ranging from atherosclerosis to nonalcoholic fatty liver disease. *Pathol. Int.* **68**, 91–101, <https://doi.org/10.1111/pin.12634>
- 194 Guo, X., Yamada, S., Tanimoto, A., Ding, Y., Wang, K.Y., Shimajiri, S. et al. (2012) Overexpression of peroxiredoxin 4 attenuates atherosclerosis in apolipoprotein E knockout mice. *Antioxid. Redox Signal.* **17**, 1362–1375, <https://doi.org/10.1089/ars.2012.4549>
- 195 Jeong, S.J., Kim, S., Park, J.G., Jung, I.H., Lee, M.N., Jeon, S. et al. (2018) Prdx1 (peroxiredoxin 1) deficiency reduces cholesterol efflux via impaired macrophage lipophagic flux. *Autophagy* **14**, 120–133, <https://doi.org/10.1080/15548627.2017.1327942>
- 196 Park, J.G., Yoo, J.Y., Jeong, S.J., Choi, J.H., Lee, M.R., Lee, M.N. et al. (2011) Peroxiredoxin 2 deficiency exacerbates atherosclerosis in apolipoprotein E-deficient mice. *Circ. Res.* **109**, 739–749, <https://doi.org/10.1161/CIRCRESAHA.111.245530>
- 197 Mowbray, A.L., Kang, D.H., Rhee, S.G., Kang, S.W. and Jo, H. (2008) Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. *J. Biol. Chem.* **283**, 1622–1627, <https://doi.org/10.1074/jbc.M707985200>
- 198 Coutinho Tde, A., Turner, S.T., Peyser, P.A., Bielak, L.F., Sheedy, II, P.F. and Kullo, I.J. (2007) Associations of serum uric acid with markers of inflammation, metabolic syndrome, and subclinical coronary atherosclerosis. *Am. J. Hypertens.* **20**, 83–89, <https://doi.org/10.1016/j.amjhyper.2006.06.015>
- 199 Acikgoz, N., Ermis, N., Yagmur, J., Muezzinoglu, K., Karakus, Y., Cansel, M. et al. (2012) Uric acid level and its association with carotid intima-media thickness in patients with cardiac syndrome X. *Med. Princ. Pract.* **21**, 115–119, <https://doi.org/10.1159/000332583>
- 200 Carvalho, L.A.C., Truzzi, D.R., Fallani, T.S., Alves, S.V., Toledo, Jr, J.C., Augusto, O. et al. (2017) Urate hydroperoxide oxidizes human peroxiredoxin 1 and peroxiredoxin 2. *J. Biol. Chem.* **292**, 8705–8715, <https://doi.org/10.1074/jbc.M116.767657>
- 201 Mani, S., Li, H., Untereiner, A., Wu, L., Yang, G., Austin, R.C. et al. (2013) Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. *Circulation* **127**, 2523–2534, <https://doi.org/10.1161/CIRCULATIONAHA.113.002208>
- 202 Bir, S.C. and Kevil, C.G. (2013) Sulfane sustains vascular health: insights into cystathionine gamma-lyase function. *Circulation* **127**, 2472–2474, <https://doi.org/10.1161/CIRCULATIONAHA.113.003489>
- 203 Bearden, S.E., Beard, Jr, R.S. and Pfau, J.C. (2010) Extracellular transsulfuration generates hydrogen sulfide from homocysteine and protects endothelium from redox stress. *Am. J. Physiol. Heart Circ. Physiol.* **299**, H1568–H1576, <https://doi.org/10.1152/ajpheart.00555.2010>
- 204 Wortmann, M., Schneider, M., Pircher, J., Hellfritsch, J., Aichler, M., Vegi, N. et al. (2013) Combined deficiency in glutathione peroxidase 4 and vitamin E causes multiorgan thrombus formation and early death in mice. *Circ. Res.* **113**, 408–417, <https://doi.org/10.1161/CIRCRESAHA.113.279984>
- 205 Ramirez, A., Ramadan, B., Ritzenthaler, J.D., Rivera, H.N., Jones, D.P. and Roman, J. (2007) Extracellular cysteine/cystine redox potential controls lung fibroblast proliferation and matrix expression through upregulation of transforming growth factor-beta. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **293**, L972–L981, <https://doi.org/10.1152/ajplung.00010.2007>
- 206 Morgan, A.R., Rerkasem, K., Gallagher, P.J., Zhang, B., Morris, G.E., Calder, P.C. et al. (2004) Differences in matrix metalloproteinase-1 and matrix metalloproteinase-12 transcript levels among carotid atherosclerotic plaques with different histopathological characteristics. *Stroke* **35**, 1310–1315, <https://doi.org/10.1161/01.STR.0000126822.01756.99>
- 207 Shah, P.K., Falk, E., Badimon, J.J., Fernandez-Ortiz, A., Mailhac, A., Villareal-Levy, G. et al. (1995) Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. Potential role of matrix-degrading metalloproteinases and implications for plaque rupture. *Circulation* **92**, 1565–1569

- 208 Okamoto, T., Akaïke, T., Sawa, T., Miyamoto, Y., van der Vliet, A. and Maeda, H. (2001) Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *J. Biol. Chem.* **276**, 29596–29602, <https://doi.org/10.1074/jbc.M102417200>
- 209 Cai, J. and Jones, D.P. (1998) Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. *J. Biol. Chem.* **273**, 11401–11404, <https://doi.org/10.1074/jbc.273.19.11401>
- 210 Janiszewski, M., Pasqualucci, C.A., Souza, L.C., Pileggi, F., da Luz, P.L. and Laurindo, F.R. (1998) Oxidized thiols markedly amplify the vascular response to balloon injury in rabbits through a redox active metal-dependent pathway. *Cardiovasc. Res.* **39**, 327–338, [https://doi.org/10.1016/S0008-6363\(98\)00082-0](https://doi.org/10.1016/S0008-6363(98)00082-0)
- 211 Tanaka, L.Y., Araujo, H.A., Hironaka, G.K., Araujo, T.L., Takimura, C.K., Rodriguez, A.I. et al. (2016) Peri/epicellular protein disulfide isomerase sustains vascular lumen caliber through an anticonstrictive remodeling effect. *Hypertension* **67**, 613–622
- 212 Muller, C., Bandemer, J., Vindis, C., Camare, C., Mucher, E., Gueraud, F. et al. (2013) Protein disulfide isomerase modification and inhibition contribute to ER stress and apoptosis induced by oxidized low density lipoproteins. *Antioxid. Redox Signal.* **18**, 731–742, <https://doi.org/10.1089/ars.2012.4577>
- 213 Tanaka, L.Y. and Laurindo, F.R.M. (2017) Vascular remodeling: a redox-modulated mechanism of vessel caliber regulation. *Free Radic. Biol. Med.* **109**, 11–21, <https://doi.org/10.1016/j.freeradbiomed.2017.01.025>
- 214 Leite, P.F., Danilovic, A., Moriel, P., Dantas, K., Marklund, S., Dantas, A.P. et al. (2003) Sustained decrease in superoxide dismutase activity underlies constrictive remodeling after balloon injury in rabbits. *Arterioscler. Thromb. Vasc. Biol.* **23**, 2197–2202, <https://doi.org/10.1161/01.ATV.0000093980.46838.41>
- 215 Focks, J.J., van Schaik, A., Clappers, N., van Dijk, E.G., van Oijen, M.G., Verheugt, F.W. et al. (2013) Assessment of plasma aminothiols levels and the association with recurrent atherothrombotic events in patients hospitalized for an acute coronary syndrome: a prospective study. *Clin. Chem. Lab. Med.* **51**, 2187–2193, <https://doi.org/10.1515/ccim-2013-0103>
- 216 Jones, D.P. (2006) Redefining oxidative stress. *Antioxid. Redox Signal.* **8**, 1865–1879, <https://doi.org/10.1089/ars.2006.8.1865>
- 217 Rossi, R., Giustarini, D., Milzani, A. and Dalle-Donne, I. (2009) Cysteinylation and homocysteinylation of plasma protein thiols during ageing of healthy human beings. *J. Cell. Mol. Med.* **13**, 3131–3140, <https://doi.org/10.1111/j.1582-4934.2008.00417.x>
- 218 Mansoor, M.A., Bergmark, C., Svardal, A.M., Lonning, P.E. and Ueland, P.M. (1995) Redox status and protein binding of plasma homocysteine and other aminothiols in patients with early-onset peripheral vascular disease. Homocysteine and peripheral vascular disease. *Arterioscler. Thromb. Vasc. Biol.* **15**, 232–240, <https://doi.org/10.1161/01.ATV.15.2.232>
- 219 El-Khairi, L., Ueland, P.M., Nygard, O., Refsum, H. and Vollset, S.E. (1999) Lifestyle and cardiovascular disease risk factors as determinants of total cysteine in plasma: the Hordaland Homocysteine Study. *Am. J. Clin. Nutr.* **70**, 1016–1024, <https://doi.org/10.1093/ajcn/70.6.1016>
- 220 Ozkan, Y., Ozkan, E. and Simsek, B. (2002) Plasma total homocysteine and cysteine levels as cardiovascular risk factors in coronary heart disease. *Int. J. Cardiol.* **82**, 269–277, [https://doi.org/10.1016/S0167-5273\(02\)00010-4](https://doi.org/10.1016/S0167-5273(02)00010-4)
- 221 Ashfaq, S., Abramson, J.L., Jones, D.P., Rhodes, S.D., Weintraub, W.S., Hooper, W.C. et al. (2006) The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults. *J. Am. Coll. Cardiol.* **47**, 1005–1011, <https://doi.org/10.1016/j.jacc.2005.09.063>
- 222 Kuhn, W., Roebroek, R., Blom, H., van Oppenraaij, D., Przuntek, H., Kretschmer, A. et al. (1998) Elevated plasma levels of homocysteine in Parkinson's disease. *Eur. Neurol.* **40**, 225–227, <https://doi.org/10.1159/00007984>
- 223 Luchsinger, J.A., Tang, M.X., Shea, S., Miller, J., Green, R. and Mayeux, R. (2004) Plasma homocysteine levels and risk of Alzheimer disease. *Neurology* **62**, 1972–1976, <https://doi.org/10.1212/01.WNL.0000129504.60409.88>

SÚMULA CURRICULAR

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1. Formação

(2014 – atual) **Doutorado direto.** Instituto do Coração, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo. Pós-graduação em Ciências pelo programa de Cardiologia.

(2009 – 2013) **Bacharelado em Biotecnologia.** Universidade Federal da Bahia, Instituto Multidisciplinar em Saúde, Campus Anísio Teixeira.

2. Experiência acadêmica

Estágio supervisionado em docência. Programa de Aperfeiçoamento de Ensino (PAE). Faculdade de Medicina, Universidade de São Paulo (02/2016 – 06/2016).
Supervisora: Naomi Kondo Nakagawa. Bolsa: PAE/FMUSP

Estágio curricular em pesquisa. Laboratório de Biologia Vascular, Instituto do Coração, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo (2013 – 2014). Projeto: Investigação da rota de externalização da dissulfeto-isomerase proteica (PDI) secretada por células vasculares. Orientador: Francisco Rafael Martins Laurindo. Co-orientadora: Thaís Larissa Araújo de Oliveira Silva. Bolsa: CAPES

Iniciação científica. Laboratório de Imunopatologia, Instituto Multidisciplinar em Saúde, Campus Anísio Teixeira, Universidade Federal da Bahia (2010 – 2013).
Projeto: Desenvolvimento de modelo experimental para o estudo de infecções pulmonares por *Ureaplasma diversum*.

Orientador: Robson Amaro Augusto da Silva. Bolsista voluntária: PIBIC/CNPq

Participação em congressos:

- (a) Functional Disulfide Bonds in Health and Disease. FASEB Meeting (2018). Apresentação oral e pôster.
- (b) EMBO Workshop Thiol oxidation in Toxicity and Signaling (2017). Elevator talk e pôster.
- (c) Functional Disulfide Bonds in Health and Disease. FASEB Meeting. (2016). Apresentação oral e pôster.

Orientações

- Isabele Nascimento Martins – Trabalho de conclusão de curso, Graduação em Biomedicina (FMU/2018). Título: CRISPR/Cas-9, a nova fronteira de edição genômica: desenvolvimento e aplicações.
- Nathalia Tengan Silva (graduação Farmácia-Bioquímica/USP) – Iniciação científica, Co-orientação (FMUSP/2018) – bolsista FAPESP). Título: Um novo ensaio de atividade da Dissulfeto Isomerase Proteica no plasma humano.
- Felipe Camilo Silva (graduação Medicina/USP) – Iniciação científica, Co-orientação (FMUSP/2018). Título: Possível papel do TMAO na homeostase redox celular de células musculares lisas.

3. Publicações

- Oliveira PVS, Garcia-Rosa S, Sachetto ATA, Moretti AIS, Debbas V, De Bessa TC, Silva NT, Pereira AC, Martins-de-Souza D, Santoro ML, Laurindo FRM. Protein Disulfide Isomerase Plasma Levels in Healthy Humans Reveal Proteomic Signatures Involved in Contrasting Endothelial Phenotypes. *Redox Biology, in press* (2019).
- De Bessa TC, Pagano A, Moretti AIS, Oliveira PVS, Mendonça SM, Kovacic H, Laurindo FRM. Subverted regulation of Nox1 NADPH oxidase-

dependent oxidant generation by protein disulfide isomerase A1 in colon carcinoma cells with overactivated KRas. *Cell Death & Disease*, 10:143, p. 1–16 (2019).

- Oliveira PVS, Laurindo FRM. Implications of plasma thiol redox in disease. *Clinical Science*, v. 132, p. 1257-1280 (2018).
- Araujo TLS, Zeidler JD, Oliveira PVS, Dias MH, Armelin HA, Laurindo FRM. Protein disulfide isomerase externalization in endothelial cells follows classical and unconventional routes. *Free Radical Biology & Medicine*, v. 103, p. 199-208 (2016).
- Silva JR, Ferreira LFAA, Oliveira PVS, Nunes IV, Pereira IS, Timenetsky J, Marques LM, Figueiredo TB, Silva RAA. Intra-uterine experimental infection by *Ureaplasma diversum* induces TNF- α mediated womb inflammation in mice. *Anais da Academia Brasileira de Ciências (Online)*, v. 88, p. 1-10 (2016).
- Oliveira PVS, Galantini MPL, Silva JR, Nunes IV, Ferreira LFAA, Pereira LAS, Marques LM, Figueiredo TB, Silva RAA. O modelo de infecção intradérmica por *Staphylococcus aureus*: uma alternativa para o entendimento do recrutamento de células para o sítio de infecção? *Scientia Plena*, v. 11, p. 1-12 (2015).