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ABBREVIATIONS

ACD – Anemia of Chronic Disease

ADP – adenosine diphosphate

AP – alternative pathway

APACHE – Acute Physiology and Chronic Health Evaluation

APC – activated protein C

aPTT – activated partial thromboplastin time

AR – absolute reduction

AT – antithrombin

BMP – Bone Morphogenetic Protein

COPD – chronic obstructive pulmonary disease

CP – classical pathway

CRP – C- reactive protein

CRRT – Continuous Renal Replacement Therapy

Del-1 – developmental endothelial locus 1

DIC – disseminated intravascular coaugulation

EC – endothelial cells

EPCR – endothelial protein C receptor

FGF – fibroblast growth factor

GAG – glycoseoaminoglycan

GEE – generalized estimating equations

GPI – glycosylphosphatidylinositol

HIT – heparin induced thrombocytopenia

HR – hazard ratio

HS – heparan sulfate

HSPG – heparan sulfate proteoglycan

ICAM 1 – intercellular adhesion molecule 1

ICU – intensive care unit

ICU-LOS – intensive care unit length of stay

IL-1 – interleukin 1

INR – international normalized ratio

IRIDA – Iron Refractory Iron Deficiency Anemia

LMWH – light molecular weight heparin

LP – lectin pathway

Mac-1 – macrophage 1 antigen

MBL – mannose binding lectin

MCP-1 – monocyte chemoattractant protein-1

MIF – macrophage migration inhibitory factor

MODS – multiple organ deficiency syndrome

NET – neutrophil extracellular trap

NF – κB – nuclear factor kappa B

NO – nitric oxide

OR – odds ratio

OS – overall survival

PAI-1 – plasminogen activator 1

PAMP – pathogen-associated molecular patterns

PAR – protease-activated receptor

PC – protein C

PDGF – platelet – derived growth factor

PDGF-BB – platelet – derived growth factor BB

PF – platelet factor

PFS – progression – free survival

PGI2 – prostaglandin I2

PMN – Polymorphonuclear cells

PolyP – polyphosphate

PRR – pattern recognition receptor

PT – prothrombin time

PTS – post – thrombotic syndrome

QIC – Quasi Likelihood Information Criterion

RAGE – receptor for advanced glycation endproducts

RCT – randomized controlled trial

RR – relative risk

SCLC – small – cell lung (/pulmonary) carcinoma

SOFA – sequential organ failure assessment

STAT3-RE – STAT3 response element

sTFR – soluble transferrin receptor

TAF – tumor – derived adhesion factor

TF – tissue factor

TFPI – tissue facto pathway inhibitor

TLR – toll-like receptor

TM – thrombomodulin

TNF – tumor necrosis factor

TNFa – tumor necrosis factor 1/alpha

UFH – unfractionated heparin

VCAM 1 – vascular cell adhesion molecule 1

VTE – venous thromboembolism

vWF – vonWillebrand factor

ΕΥΧΑΡΙΣΤΙΕΣ

Για την παρούσα διατριβή, πρωτίστως θα ευχαριστήσω τους ασθενείς που συμμετείχαν στην έρευνα, καθώς και τους συγγενείς τους, που μέσα στα σοβαρά προβλήματα που βασάνιζαν τους ίδιους ή τα αγαπημένα τους πρόσωπα, αντίστοιχα, βρήκαν το ψυχικό σθένος να μας παραχωρήσουν τη συγκατάθεσή τους για να τους εντάξουμε στην έρευνα και να τους μελετήσουμε, με στόχο την πρόοδο της ιατρικής επιστήμης. Ποτέ δεν πρέπει να ξεχνάμε ότι αυτοί είναι πάντα το επίκεντρο του μαγικού μας λειτουργήματος.

Έπειτα θα ευχαριστήσω τα μέλη της επταμελούς επιτροπής, για την αξιολόγηση της διατριβής μου και την κριτική τους που μου επιτρέπει να την παρουσιάσω στη βέλτιστη μορφή. Μια ξεχωριστή ευχαριστία αρμόζει στα μέλη της τριμελούς επιτροπής μου που με καθοδηγούσαν καθ' όλη τη διάρκεια της πορείας μου ως υποψήφιος διδάκτωρ. Συγκεκριμένα, ευχαριστώ την κ.Κουτσούκου, που επέτρεψε να αντλήσω το δείγμα ασθενών μας από τη Μονάδα Εντατικής Θεραπείας που διεύθυνε και για τη μεθοδικότητα με την οποία τους παρείχε θεραπεία. Έπειτα, ευχαριστώ την κ.Πολίτου, που μελέτησε στο αιματολογικό εργαστήριο τα δείγματα των ασθενών μας, αλλά κυρίως για την από κοινού με τον επιβλέποντα κ.Βασιλειάδη σύλληψη του θέματος της διατριβής μου, ένα θέμα το οποίο έχει μόλις την τελευταία δεκαετία απασχολήσει την ιατρική κοινότητα.

Το μεγαλύτερο όμως «ευχαριστώ» ανήκει αδιαμφισβήτητα στον επιβλέποντα καθηγητή μου, κ.Βασιλειάδη. Η καθοδήγηση και η στήριξη που μου προσέφερε στα χρόνια της άψογης συνεργασίας μας, με κάνουν να τον βλέπω όχι μόνο ως ένα εκ των ων ουκ άνευ κομμάτι της προόδου μου, αλλά ως ένα πρότυπο και κυρίως ως ένα σημαντικό μου φίλο.

ΟΡΚΟΣ ΙΠΠΟΚΡΑΤΗ

Ὄμνυμι Ἀπόλλωνα ἰητρὸν, καὶ Ἀσκληπιὸν, καὶ Ὑγείαν, καὶ Πανάκειαν, καὶ θεοὺς πάντας τε καὶ πάσας, ἵστορας ποιεύμενος, ἐπιτελέα ποιήσειν κατὰ δύναμιν καὶ κρίσιν ἐμὴν ὅρκον τόνδε καὶ ξυγγραφὴν τήνδε.

Ἡγήσασθαι μὲν τὸν διδάξαντά με τὴν τέχνην ταύτην ἴσα γενέτῃσιν ἐμοῖσι, καὶ βίου κοινώσασθαι, καὶ χρεῶν χρηίζοντι μετάδοσιν ποιήσασθαι, καὶ γένος τὸ ἐξ ωὐτέου ἀδελφοῖς ἴσον ἐπικρινέειν ἄῤῥεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἢν χρηίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ ξυγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἁπάσης μαθήσιος μετάδοσιν ποιήσασθαι υἱοῖσί τε ἐμοῖσι, καὶ τοῖσι τοῦ ἐμὲ διδάξαντος, καὶ μαθηταῖσι συγγεγραμμένοισί τε καὶ ὡρκισμένοις νόμῳ ἰητρικῷ, ἄλλῳ δὲ οὐδενί.

Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν.

Οὐ δώσω δὲ οὐδὲ φάρμακον οὐδενὶ αἰτηθεὶς θανάσιμον, οὐδὲ ὑφηγήσομαι ξυμβουλίην τοιήνδε. Ὁμοίως δὲ οὐδὲ γυναικὶ πεσσὸν φθόριον δώσω. Ἁγνῶς δὲ καὶ ὁσίως διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμήν.

Οὐ τεμέω δὲ οὐδὲ μὴν λιθιῶντας, ἐκχωρήσω δὲ ἐργάτῃσιν ἀνδράσι πρήξιος τῆσδε.

Ἐς οἰκίας δὲ ὁκόσας ἂν ἐσίω, ἐσελεύσομαι ἐπ' ὠφελείῃ καμνόντων, ἐκτὸς ἐὼν πάσης ἀδικίης ἑκουσίης καὶ φθορίης, τῆς τε ἄλλης καὶ ἀφροδισίων ἔργων ἐπί τε γυναικείων σωμάτων καὶ ἀνδρῴων, ἐλευθέρων τε καὶ δούλων.

Ἃ δ' ἂν ἐν θεραπείῃ ἢ ἴδω, ἢ ἀκούσω, ἢ καὶ ἄνευ θεραπηίης κατὰ βίον ἀνθρώπων, ἃ μὴ χρή ποτε ἐκλαλέεσθαι ἔξω, σιγήσομαι, ἄῤῥητα ἡγεύμενος εἶναι τὰ τοιαῦτα.

Ὅρκον μὲν οὖν μοι τόνδε ἐπιτελέα ποιέοντι, καὶ μὴ ξυγχέοντι, εἴη ἐπαύρασθαι καὶ βίου καὶ τέχνης δοξαζομένῳ παρὰ πᾶσιν ἀνθρώποις ἐς τὸν αἰεὶ χρόνον. παραβαίνοντι δὲ καὶ ἐπιορκοῦντι, τἀναντία τουτέων.

ΒΙΟΓΡΑΦΙΚΟ

Προσωπικά στοιχεία

ΤΟΠΟΣ ΓΕΝΝΗΣΗΣ: Θεσσαλονίκη
ΗΜΕΡΟΜΗΝΙΑ ΓΕΝΝΗΣΗΣ: 22/10/1991
ΥΠΗΚΟΟΤΗΤΑ: Ελληνική

Εκπαίδευση

- *Ιούλιος 2019-Σήμερα* **Υποψήφιος Διδάκτωρ** τμήματος **Ιατρικής** Σχολής Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών (ΕΚΠΑ), με θέμα «Μελέτη της Πλειοτροπικής Δράσης της Αντιπηκτικής Αγωγής σε Βαρέως Πάσχοντες Ασθενείς»

- *Φεβρουάριος 2019* **Μεταπτυχιακό** Δίπλωμα Ειδίκευσης, από το τμήμα **Ιατρικής** Σχολής Αθηνών (ΕΚΠΑ), με τίτλο «Καρδιοπνευμονική Αποκατάσταση και Αποκατάσταση Πασχόντων ΜΕΘ» Βαθμός: Άρ**ι**στα – 8.65

- *Ιούλιος 2016* Πτυχίο **Ιατρικής,** από το τμήμα Ιατρικής Σχολής Αριστοτελείου Πανεπιστημίου Θεσσαλονίκης (ΙΣΑΠΘ) Βαθμός: Λίαν Καλώς – Επτά

- *Ιούνιος 2009* Απολυτήριο Γενικού Λυκείου από το Πειραματικό Σχολείο Αριστοτελείου Πανεπιστημίου Θεσσαλονίκης (ΠΣΑΠΘ) Βαθμός: Άριστα – Δεκαεννιά & τρία δέκατα

Επαγγελματική εμπειρία

- *Οκτ. 2020-Σήμερα* Ειδικευόμενος Αναισθησιολογίας στο 251 Γενικό Νοσοκομείο Αεροπορίας, Αθήνα

- *Σεπ. 2017 – Σεπ. 2020* Ιατρός (Υποσμηναγός) στην 117 Πτέρυγα Μάχης, Ανδραβίδα

- *Οκτ. 2016 – Σεπ. 2017* Ιατρός (Ανθσγός) στο 251 Γενικό Νοσοκομείο Αεροπορίας, Αθήνα

- *Σεπ. 2016* Εκπαιδευόμενος ιατρός στην Πνευμονολογική Κλινική του 424 Γενικού Στρατιωτικού Νοσοκομείου Εκπαιδεύσεως, Θεσσαλονίκη

- *Σεπ. 2009 – Οκτ. 2016* Μαθητής στη Στρατιωτική Σχολή Αξιωματικών Σωμάτων (ΣΣΑΣ)

Επιστημονικές εργασίες

- Βιβλιογραφική ανασκόπηση ως **πρώτος συγγραφέας** στο περιοδικό **In Vivo** (Impact Factor: 2.35) με τίτλο "Thromboinflammation in Sepsis and Heparin: A Review of Literature and Pathophysiology", Σεπτέμβριος 2022.
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- Δημοσίευση πρωτότυπης έρευνας στο περιοδικό **Health and Research Journal** με τίτλο "Addition of strength training modifies the aerobic exercise inflammatory response in heart failure patients–Comments on the underlying pathophysiology", Απρίλιος 2022.
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Ειδική επιμόρφωση

- Ολοκλήρωση του προγράμματος εκπαίδευσης **Advanced Trauma Life Support® (ATLS®)**, στο 251 Γενικό Νοσοκομείο Αεροπορίας, Δεκέμβριος 2018.

- Ολοκλήρωση του προγράμματος εκπαίδευσης **Prehospital Trauma Life Support® (PHTLS®)**, στο 251 Γενικό Νοσοκομείο Αεροπορίας, Ιανουάριος 2017. Αναμένεται από τον ίδιο φορέα ο ορισμός ως **instructor** στα μελλοντικά προγράμματα.

- Ολοκλήρωση του σεμιναρίου **Προχωρημένης Καρδιοπνευμονικής Αναζωογόνησης** στο 251 Γενικό Νοσοκομείο Αεροπορίας, Απρίλιος 2017.

- Παρακολούθηση του 1ου Εκπαιδευτικού Σεμιναρίου Κλινικής Αεροπορικής Ιατρικής, 251 ΓΝΑ, 20-22/2/2017.

- Παρακολούθηση του 26ου Πολυθεματικού Ιατρικού Συμποσίου, 251 ΓΝΑ, 22/2/2017.

- Παρακολούθηση του 7ου Πανελλήνιου Συνεδρίου Φαρμακολογίας**,** Θεσσαλονίκη, 18-20/5/2012,.

- Παρακολούθηση του 23ου Ιατρικού Συνεδρίου Ενόπλων Δυνάμεων, Θεσσαλονίκη, 4-7/11/2010.

<u>Στρατιωτικά Σχολεία</u>

- Αποφοίτηση από το **Σχολείο Προσομοιωτή Διαφυγής από Βυθιζόμενο Ελικόπτερο** (502η σειρά HELO DUNKER), Σχολή Ελικοπτέρων Ναυτικού, Μάιος 2017.

- Αποφοίτηση από το **Σχολείο Αεροπορικής Ιατρικής** (49η σειρά του ΣΑΙ), Κέντρο Αεροπορικής Ιατρικής, Μάρτιος 2017.

<u>Ξένες γλώσσες</u>

- **Αγγλικά:** Άριστη γνώση (επίπεδο C2)
  (Certificate of Proficiency in English, University of Cambridge)
  (Certificate of Proficiency in English, University of Michigan)

- **Γερμανικά:** Πολύ καλή γνώση (επίπεδο C1)
  ("Mittelstufe" Zertifikat C1, Goethe Institut)

- **Γαλλικά:** Ενδιάμεση γνώση (επίπεδο B1)
  (DELF B1 Diplôme d'études en langue française, Commision nationale du DELF et du DALF)

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Υπόβαθρο: Παρά την πρόοδο στα πρωτόκολλα μηχανικού αερισμού, ενυδάτωσης και στην αντιβιοτική αγωγή, τα ποσοστά επιβίωσης στη Μονάδα Εντατικής Θεραπείας παραμένουν απαράδεκτα χαμηλά. Η σήψη παραμένει ένα μείζον αίτιο υψηλής θνητότητας μεταξύ των βαρέως πασχόντων ασθενών. Η βασική παθοφυσιολογία της σήψης χαρακτηρίζεται από αλληλεπίδραση μεταξύ πηκτικότητας και φλεγμονής. Οπότε, παράγοντες που μετριάζουν και τα δύο πιθανόν να βελτιώνουν τα αποτελέσματα. Η ηπαρίνη είναι ένας τέτοιος παράγοντας, αφού, πέρα από την ευρύτατα γνωστή αντιπηκτική της ιδιότητα, επίσης ασκεί ανοσοτροποποιητική δράση και προστατεύει το γλυκοκάλυκα. Για την ακρίβεια, το εύρος της πλειοτροπικής δράσης των ηπαρινών είναι τόσο εκτεταμένο, που ο χαρακτηρισμός τους αποκλειστικά ως αντιπηκτικά είναι υποτίμηση, καθώς κατέχουν ιστο-προστατευτικές, νευρο-προστατευτικές, νεφρο-προστατευτικές, καρδιο-προσταευτικές ιδιότητες και επιπλέον ασκούν αντι-καρκινική, αντι-μεταστατική, αντι-αθηρωτική και τέλος, αντι-φλεγμονώδη και anti-hepcidin δράση. Η ισχυρή anti-hepcidin ιδιότητα της ηπαρίνης είναι ένα αντικείμενο μελέτης το οποίο μόλις πρόσφατα έχει ερευνηθεί κατά την τελευταία δεκαετία, μολονότι όχι επαρκώς σε ανθρώπους, πόσο δε μάλλον σε βαρέως πάσχοντες. Η hepcidin είναι ο βασικός ρυθμιστής της ομοιόστασης του σιδήρου. Οι τρεις κύριες πηγές διαθεσιμότητας σιδήρου είναι η διαιτητική πρόσληψη, η ανακύκλωση ερυθροκυττάρων και οι σωματικές αποθήκες σιδήρου και παρότι ο σίδηρος είναι ουσιώδης για τη ζωή, είναι επίσης δυνητικά τοξικός. Η περίσσεια της hepcidin οδηγεί σε μείωση των επιπέδων σιδήρου, όπως παρατηρείται στη Σιδηροπενική Αναιμία την Ανθεκτικη στη Θεραπεία με Σίδηρο (IRIDA), καθώς και στην Αναιμία της Φλεγμονής (ή Αναιμία της Χρόνιας Νόσου), κοινές σε έναν αξιοσημείωτο αριθμό εκφυλιστικών νόσων. Η anti-hepcidin δράση της ηπαρίνης φαίνεται να εξαρτάται σε ένα μεγάλο βαθμό από ένα υψηλό μοριακό βάρος. Υποθέσαμε λοιπόν ότι η μη – κλασματοποιημένη ηπαρίνη, με το μεγάλο μοριακό της βάρος, θα μείωνε αποτελεσματικά την έκφραση της hepcidin μεταξύ των βαρέως πασχόντων ασθενών. Από όσο γνωρίζουμε, αυτή είναι η πρώτη μελέτη που διεξήχθη σε ανθρώπους.

Σκοπός: Να διευκρινιστεί αν η μη – κλασματοποιημένη ηπαρίνη επιφέρει μείωση των επιπέδων της hepcidin στους βαρέως πάσχοντες ασθενείς.

Σχεδιασμός: Προοπτική, μη – επεμβατική μελέτη παρατήρησης, διεξαχθείσα κατά την περίοδο του Οκτωβρίου 2017 μέχρι Δεκέμβριο 2019.

Περιβάλλον: Μονάδα Εντατικής Θεραπείας 10 κλινών της Α' Πανεπιστημιακής Πνευμονολογικής Κλινικής του Γενικού Νοσοκομείου Νοσημάτων Θώρακος «Η Σωτηρία».

Συμμετέχοντες: 22 βαρέως πάσχοντες ασθενείς, εκ των οποίων οι 16 ήταν σηπτικοί. Κριτήρια εκλογής ήταν νοσηλεία διάρκειας τουλάχιστον πέντε ημερών και χορήγηση μη – κλασματοποιημένης ηπαρίνης, χορηγηθείσας μόνο κατά την κρίση του θεράποντος ιατρού για οποιονδήποτε λόγο.

Έκθεση: Χορήγηση μη – κλασματοποιημένης ηπαρίνης στην $1^{η}$, $2^{η}$ και $5^{η}$ ημέρα νοσηλείας.

Κύριο Αποτέλεσμα και Μετρήσεις: Τα μέσα επίπεδα της hepcidin ήταν σημαντικά μειωμένα σε σχέση με τα επίπεδα πριν την έναρξη θεραπείας έπειτα ήδη από την $1^{η}$ ημέρα χορήγησης ηπαρίνης (p=0.003).

Αποτελέσματα: Η μέση ηλικία των ασθενών (SD) ήταν 72.6 (9,6) έτη και το BMI ήταν 30,1 (6,7) kg/m^2. Η μέση διάρκεια νοσηλείας στη ΜΕΘ ήταν 13 (5,8 – 26,8) ημέρες (διατερταμοριακό εύρος). Η θνητότητα εντός της ΜΕΘ ήταν 27,3% (95% confidence interval 17,1 – 47,5) και το μέσο APACHE II σκορ κατά την εισαγωγή ήταν 24,5 (9,8). Η ηπαρίνη επέδειξε μια ισχυρή ανεξάρτητη αρνητική συσχέτιση με τη hepcidin (p<0,001). Μια εκτιμώμενη μείωση των επιπέδων της hepcidin κατά 375 έως 539 pg/ml αναμένεται για κάθε 1000 IU αύξηση στη δόση της χορηγούμενης ηπαρίνης. Ένα επιπρόσθετο εύρημα ήταν η ανεξάρτητη θετική συσχέτιση της κρεατινίνης με τη hepcidin (μια εκτιμώμενη αύξηση των επιπέδων της hepcidin κατά 3645 έως 4783 pg/ml αναμένεται για κάθε αύξηση των επιπέδων κρεατινίνης κατά 1 mg/dl).

Συμπεράσματα και Σχετικότητα: Η αντι – hepcidin ιδίοτητα των ηπαρινών, η οποία έχει δείξει ότι συναρτάται με το μοριακό τους βάρος, επιβεβαιώθηκε για πρώτη φορά σε ένα δείγμα ασθενών αποτελούμενο αποκλειστικά από ανθρώπους. Αυτό ενδέχεται να οδηγήσει σε

μελλοντικές θεραπευτικές μεθόδους τύπων αναιμίας που χαρακτηρίζονται από περίσσεια hepcidin, κοινές μεταξύ των βαρέως πασχόντων.

Λέξεις – κλειδιά:

Ηπαρίνη• ανοσοθρόμβωση• σήψη• Hepcidin• βαρέως πάσχοντες• Μονάδα Εντατικής Θεραπείας•

Title: Study of the pleiotropic effect of anticoagulant treatment on critically ill patients

ABSTRACT

Background: Despite advancements in mechanical ventilation protocols, fluid resuscitation and antibiotic treatment and maintenance of homeostatic blood glucose, survival rates in the Intensive Care Units still remain unacceptably low. Sepsis is a major cause of high mortality among critically ill patients. The basic pathophysiology of sepsis is characterized by interaction between coagulation and inflammation. Therefore, agents that ameliorate both may improve outcome. Heparin is such an agent, since, beyond the well – known anticoagulant property, it also exerts immunomodulatory, glycocalyx – protective activity. In fact, the range of the pleiotropic effect of heparin is so wide, that characterizing it solely as an anticoagulant is an underestimation, since it possesses tissue – protective, neuro – protective, nephro – protective, cardiovascular – protective properties and they further exert anti – cancer and anti – metastatic activity, anti – atherosclerotic activity and finally, anti – inflammatory and anti – hepcidin activity. Its strong anti – hepcidin effect is a subject of study that has only just recently been investigated during the past decade, albeit not adequately among humans, even more so among critically ill patients. Hepcidin is the basic regulator of iron homeostasis. The three main sources providing iron bioavailability are dietary absorption, red blood cell recycling and body iron reserve and, even though it is essential for life, it's also potentially toxic. Excessive hepcidin results to iron decrease, noticed in Iron Refractory Iron Deficiency Anemia, as well as Anemia of Inflammation (or Anemia of Chronic Disease), common in a non-negligible number of degenerative diseases. Heparin's anti – hepcidin activity seems highly dependent on a high molecular weight. We therefore hypothesized that unfractionated heparin, with its high molecular weight, would effectively repress hepcidin expression amongst critically ill patients. To the best of our knowledge, this is the first study to be conducted on humans.

Aim – Objective: To determine whether unfractionated heparin leads to decrease of hepcidin levels in critically ill patients.

Design: Prospective, non-invasive, observational study, conducted over the course of October 2017 and December 2019.

Setting: A 10-bed Intensive Care Unit (ICU) of the 1st Department of Respiratory Medicine, Sotiria Thoracic Diseases Hospital

Participants: 22 critically ill patients, out of which 16 septic. Inclusion criteria were a hospitalization of at least 5-day duration and administration of unfractionated heparin, prescribed only at the attending physician's discretion for any reason.

Exposures: Administration of unfractionated heparin on the 1st, 2nd and 5th day of hospitalization.

Main Outcomes and Measures: Mean hepcidin levels were significantly reduced compared to baseline following the 1st day of heparin administration (p=0.003).

Results: Mean patient age (SD) was 72.6 (9.6) years and BMI was 30.1 (6.7) kg/m^2. Median ICU length of stay was 13 (5.8 – 26,8) days (interquartile range). ICU mortality was 27.3 % (95% confidence interval 17.1 – 47.5) and mean APACHE II score on admission was 24.5 (9.8). Heparin displayed a strong independent negative association with hepcidin (p <0.001). An estimated decrease of hepcidin levels by 375 to 539 pg/ml is expected for every 1000 IU increase in heparin dose administered. An additional finding was creatinine's independent positive association with hepcidin (an estimated increase of hepcidin levels by 3645 to 4783 pg/ml is expected for every 1 mg/dl increase in creatinine levels).

Conclusions and Relevance: The anti-hepcidin effect of heparins, which has been shown to rely on their molecular weight, was confirmed for the first time in a sample of humans. This may lead to future treatment methods of forms of anemia characterized by an excess of hepcidin, common amongst the critically ill.

Key words:

Heparin; immunothrombosis; sepsis; hepcidin; critical illness; Intensive Care Unit;

PROLOGUE – INTRODUCTION

Hepcidin is the basic hormone regulating iron bioavailability from three main sources: dietary absorption, red blood cell recycling and body iron reserve. Hepcidin acts by causing degradation of ferroportin, the main exporter of iron to the extracellular matrix, thus preventing iron release into the bloodstream and leading to the binding of iron in duodenal enterocytes, macrophages and hepatocytes. Iron level increase stimulates production of Hepcidin in order to prevent further exportation of iron to the bloodstream and to avert excessive iron accumulation. Inflammation also promotes Hepcidin production to reduce iron accessibility to pathogens. Heparin may inhibit hepcidin production by preventing BMP-SMAD signaling. However, the anticoagulant properties of heparin are an obvious restriction for its possible therapeutic application in treatment of iron disorders. Chemical modifications of heparin without anticoagulant activity, that are obtained by reduction and oxidation with glycol are the so called glycol-split heparins, that retain their ability to inhibit the BMP signaling pathway and prevent hepcidin production in the liver both in vitro and in vivo. GS-heparins are a new possible therapeutic means to target the respective signaling pathways that regulate hepcidin regulation and to treat inflammatory anemia. Despite the aforementioned progress in the study of this protein molecule's effect, there is no evidence concerning the fluctuation of hepcidin levels in critically ill patients that are hospitalized in Intensive Care Units and are treated with heparin. The primary target of the study described in the present thesis was the correlation of hepcidin levels with administered heparin but also with changes in hemoglobin concentration levels in patients nursed in the ICU and a possible correlation with clinical symptoms and markers of Systemic Inflammatory Response Syndrome (clinical and laboratory indicators, such as CRP or/and IL-6), regardless of the cause. The study was conducted in a 10-bed ICU of "Sotiria" Hospital. Special thanks are due to professor Vasileiadis for his consistent guidance and invaluable support throughout the years of our cooperation that have led me to consider him not only an integral part of my progress, but also an invaluable friend.

ΓΕΝΙΚΟ ΜΕΡΟΣ

THEORETICAL CONTEXT

1. Brief description of heparin

Heparin is one of the oldest agents still widely clinically administered thanks to its coagulation inhibiting and venous thromboembolism (VTE) preventing ability. This naturally sulfated polysaccharide displays the strongest negative charge among all biological molecules and displays a molecular weight varying from 3 to as high as 30 kDa. Its discovery is attributed to Jay McLean and William Henry Howell, who in 1916 researched the substances responsible for the clotting of blood. As it was extracted from canine brain, it was originally termed as "cephalin", but was coined as "heparin" by Howell no later than 1918, a name deriving from the Greek term for liver ("ήπαρ"), being isolated since then from canine liver. (Wardrop and Keeling, 2008) In 1939, Roche – Organon developed from bovine lung the first pharmaceutical preparations to ever be commercialized in the United States and then to be replaced by a preparation isolated from porcine mucosa (Torri and Naggi, 2016). However, it took almost another 30 years for the mechanism of the anticoagulant activity of heparin to be unraveled, linked with a high binding affinity to antithrombin (AT) of a pentasaccharide, specifically AT – bs (Antithrombin III binding site), present only in one third of the heparin molecule chain, recognized by the sequence: GclNAc6SO3–GlcA-GlcNSO3-6SO3–IdoA2SO3-GlcNSO3-6SO3.

Following the 1970s decade, work has been conducted by many groups to fractionate and depolymerize the molecule to create low molecular weight heparins (LMWH), or to generate unfractionated heparins or, alternatively, to otherwise modify it and produce derivatives with no activity on coagulation. Heparin's biological function is not attributed to its anticoagulant property, but rather to its ability to interact with numerous proteins; both early and recent studies have demonstrated that heparins display tissue – protective, neuro – protective, nephro – protective, cardiovascular – protective properties and they further exert anti – cancer and

anti – metastatic activity, anti – atherosclerotic activity and finally, anti – inflammatory and anti – hepcidin activity (Cassinelli and Naggi, 2016). The latter two seem to also be exerted among critically ill patients, as heparin has shown to display not only an anti-inflammatory, but an anti-thromboinflammatory effect, i.e. properties combating thromboinflammation, the pathological condition of generalized immunothrombosis, a physiological response in which coagulation and inflammation interact and complement each other, observed in a non-negligible number of degenerative diseases, one of them being sepsis. The anti – hepcidin activity of heparin, previously studied more rigorously in vitro and mice, seems to also apply to critically ill patients and will be analyzed further below.

2. The 100-year-old pleiotropic activity of heparin

2.1. Heparin's anti – inflammatory property

The discovery of heparin's anti – inflammatory effects sparked much interest and anticipation towards the possible development of agents similar to heparin, spared of its anti – coagulatory activity.

For comprehending heparin's pharmacology, recognition of the source substance, the heparan sulfate (HS) proteoglycans, is in order. All tissues include HS proteoglycans associated with the cell surface, the enveloping glycocalyx and basement membranes. On the quiescent endothelium, these HS proteoglycans act as natural anticoagulants and inhibit thrombosis development on the intact interior layer of the vessels (Mertens *et al.*, 1992). These endogenous heparans possess a wide variety of biological effects, e.g. harboring proteins like lipoprotein lipase and facilitating transmembrane transport function (Li and Vlodavsky, 2009). They play an essential role in the triggering and the preservation of the inflammatory cascade. The expression of selectins and other leukocyte adhesion molecules on EC surfaces propagates the continuity of the initial inflammatory reaction through recruitment of circulating leukocytes, which are then able to permeate and/or trans-migrate the vessel walls (Wang *et al.*, 2002; Li and Vlodavsky, 2009). HS proteoglycans also interact with several inflammatory cytokines, such as IL-2, IL-8 and IL-10 (Najjam *et al.*, 1998; Spillmann, Witt and Lindahl, 1998; Salek-Ardakani *et al.*, 2000; Li and Vlodavsky, 2009).

Heparin possesses a biological basis as a regulator of inflammation and its anti – inflammatory properties take place at several levels: firstly, heparin hinders neutrophil activation and function. This process is initiated with selectin expression inhibition, restraining neutrophil recruitment into the tissues (Tichelaar, Kluin-Nelemans and Meijer, 2012). Heparin also interrupts neutrophil activity through the prevention of the function of the neutrophil proteases, cathepsin G and leukocyte elastase, which are able to propagate inflammation in the adult respiratory distress syndrome and in cystic fibrosis (Wakefield *et al.*, 1993).

Secondly, heparin, via its interaction with the endothelium, inhibits the expression of inflammatory mediators which trigger lead the activation of the innate immune system. These interactions involve the reduction of the translocation of transcription factor nuclear factor – kappaB (NF-κB) from the cytoplasm into the nucleus (Thourani *et al.*, 2000), as well as reduction of IL-6, IL-8, IL-1 beta and TNF – alpha (Hochart *et al.*, 2006). Heparin has been shown, in both animal and human studies, to lower TNF – alpha activity (Salas *et al.*, 2000) and hinders the activation of the receptor for advanced glycation endproducts (RAGE) (Rao *et al.*, 2010). Interactions with CD11b, which is a major mediator of the innate immune response, direct the impact of heparin on these two molecules.

Thirdly, heparin prevents vascular smooth muscle cell proliferation (Gilotti *et al.*, 2014). Since the latter can gradually lead to flow – suppressing stenosis, heparin may play a role in inhibiting the progression of arterial disease. In venous system diseases, however, this mechanism has a lower extent, since vascular smooth muscle proliferation contributes less to venous inflammation pathology.

Finally, heparin prevents inflammation through its anticoagulant activity. Inflammation and thrombosis engage in close interplay and inhibition of thrombosis can therefore ameliorate inflammation; this process will be discussed in extent further below. A reduction in the formation of thrombin reduces in turn production of intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1) and macrophage migration inhibitory factor (MIF) by ECs. It also lowers thrombin – induced EC permeability in a PAR-1-dependent fashion, as well as thrombin – dependent platelet activation (Gonzales *et al.*, 2014).

A great number of studies have shown the anti – inflammatory properties of heparin. And still, not so much is known about the differences between various heparin preparations and their anti – inflammatory activity. Deleting or inactivating the AT III binding domain selectively leads to non – anticoagulant preparations of heparin. Despite the removal of their anticoagulant effect, however, these heparins retain their anti – inflammatory activity (Gao *et al.*, 2005; Rao *et al.*, 2010). Heparin's ability to suppress inflammation may be attributed to domains of its

polysaccharide sequence besides the AT III binding site. This observation indicates that not all heparins display the same non – anticoagulant functions, as a result of differentiations in the structure and length of their polysaccharide chains.

Taking into account the greater size of its polysaccharide chains, UFH could be presumed to possess increased anti – inflammatory activity in comparison with LMWH. This is not the case, however, as LMWH anti – inflammatory function is at the very least as strong as that of UFH. In a study of cultured monocytes (Hochart *et al.*, 2006), both LMWH and UFH were evaluated for their lipopolysaccharide – induced cytokine production. Both heparins ameliorated to the same extent the inflammatory response, defined by measurements of TNF-alpha, IL-1 beta, IL-6, IL-8 and NF-κB.

In a mouse model with experimental VTE (Downing *et al.*, 1998), both LMWH and UFH were assessed at low and high doses. Only in the LMWH arm was an inflammation reduction evident. This activity was not reliant on the achievement of an anticoagulatory state, suggesting that heparins apply anti – inflammatory effects that are not anticoagulation – dependent. Similar conclusions were reached in a study including hemodialysis patients (Poyrazoglu *et al.*, 2006). LMWH, but not UFH, led to a reduction of oxidative stress and inflammation systemic markers.

The smallest heparinoid in clinical use currently is fondaparinux, which consists of only the pentasaccharide AT III binding site, common to both UFH and LMWH. In one study (Frank *et al.*, 2005), fondaparinux was shown to improve survival rates and to mute the systemic inflammatory response in mice with renal hypoxia caused by a temporary interruption of renal blood flow. In a follow – up study (Frank *et al.*, 2006), fondaparinux was chemically altered in order to delete its affinity for AT, therefore muting its anticoagulant effect. The resulting synthetic, non – anticoagulant pentasaccharide preserved its anti – inflammatory property in the same mouse preparations, indicating that even short polysaccharide chains may downplay inflammation and these non – anticoagulant heparins may prove to be treatment modalities for targeted anti – inflammatory treatment.

Some of the properties unrelated to coagulation are believed to be the corollary of the connection of cytokines and heparan sulfate-dependent growth factors with heparin, next to

other agents, as for example cytotoxic peptides, molecules that regulate cell adhesion and degradation enzymes. This binding might attenuate these proteins' activity, therefore inhibiting tissue damage and cell activation (Fredens, Dahl and Venge, 1991; Page, 1991; Walsh *et al.*, 1991; Gilat *et al.*, 1994; Bono *et al.*, 1997). However, the exact processes through the anti – inflammatory actions are exerted are as of yet not fully elucidated.

Heparin can disrupt the inflammatory process through numerous ways. By binding to and inhibiting enzymes and mediators of inflammation, heparin prevents inflammatory cell activation. Moreover, this action further inhibits substances that would be secreted by these cells, assisting as such dissemination of inflammation; remodeling and tissue damage are additionally prevented by heparin (Handel *et al.*, 2005; Brown *et al.*, 2006; Lever *et al.*, 2007).

Additionally, heparin hinders mast cell degranulation, attenuating the production of histamine and suppressing the cytotoxicity exerted by activated eosinophils versus endothelial cells (EC). Activation of leukocytes and their adhesion are pivotal in the inflammatory response process. Excessive activation of leukocytes leads to intravascular aggregation and production of proteolytic enzymes and free radicals, which in turn propagate endothelial damage. Evidently, heparin disrupts both leukocyte activation and aggregation. In vitro as well as in vivo studies suggest that heparin hinders the transfer of leukocytes through the subendothelial basement membrane, which downplays adhesion molecule expression and pro – inflammatory agent production (Johnson *et al.*, 2004).

Finally, heparin prevents platelet – released heparinase, an agent which increases migration of leukocytes. The activation of platelets partakes in both the intravascular coagulation and the inflammatory mechanisms via P – Selectin expression, a strong agent of inflammation, expressed on endothelial surfaces, monocytes and neutrophils. Upon heparin incubation with neutrophils, adhesion of those cells to the endothelial cell stimulated by platelet thrombin or activating factors is inhibited (Greinacher, 2011). The role of heparin, not only in inflammation, but also in inflammation's cross – talk with the coagulation cascades will be discussed in extent further below.

2.2. Heparin and inflammatory diseases

Through the course of the past decade, an increasing number of clinical studies have been conducted, examining the effects of heparin among patients suffering from asthma, rhinitis and chronic obstructive pulmonary disease (COPD), the latter being a very common disease amongst the critically ill, having been found in a study to affect 8.6 % of all of the patients admitted to the ICUs and complicating the affected patients' survival rates since, in comparison with patients without COPD, the adjusted mortality, as calculated by the ratio of observed to expected mortality, was increased among patients admitted because of acute respiratory failure due to COPD and even more so among patients with comorbid COPD (Funk *et al.*, 2013)

2.2.1. Asthma

Asthma, a chronic disease with no cure, characterized by obstruction of airflow, affects an estimate of 300 million people worldwide. Patients suffer a state of permanent allergic inflammation in the airways which usually leads to a progressively diminished pulmonary function. Modifications within the bronchoalveolar space, e.g. plasma protein extravasation and coagulation mediator presence within this microenvironment are common findings of lung diseases (de Boer *et al.*, 2012).

Numerous placebo – controlled studies of small size have evaluated the effect of heparin in inhaled form, heparin – derived agents or LMWH on asthmatic patients' airway tissues, presenting evidence of reduction in early and late bronchial response (Duong *et al.*, 2008), hyperreactivity reduction (Stelmach *et al.*, 2003) and a decrease in the number of inflammatory cell present in bronchoalveolar lavage fluid (Fal *et al.*, 2001; Passowicz-Muszyńska, Jankowska and Krasnowska, 2002).

Evidence of nebulized heparin's protective effect on bronchoconstriction was additionally presented in a double – blind, placebo – controlled trial (Tutluoğlu *et al.*, 2001). Respiratory function tests were conducted, previous to and following KCl 10% inhalation. Despite an FEV1

reduction of 16.4% in the control group, patients treated with heparin before hypertonic KCl inhalation demonstrated almost no change regarding that particular parameter.

2.2.2. Rhinitis

Among patients with rhinitis, heparin has demonstrated to significantly downplay symptomatology and eosinophil presence on nasal lavage fluid (Vancheri *et al.*, 2001)

2.2.3. Chronic Obstructive Pulmonary Disease

In COPD, systemic inflammation is observed, marked by progressive airflow limitation and dyspnea, remodeling of the airways and ultimately, destruction of the lung parenchyma leading to emphysema (Cockayne *et al.*, 2012). Even though it is a chronic disease, acute periods of exacerbation of symptomatology may occur, which are a major component of the disease's clinical course, as they not only become more frequent as the disease progresses but also because they are considered as milestones in the affected patients' lives (Paggiaro *et al.*, 1998; Miravitlles *et al.*, 1999, 2000; Burge *et al.*, 2000; Greenberg *et al.*, 2000; Gompertz *et al.*, 2001; Donaldson *et al.*, 2002; Halpern, Stanford and Borker, 2003).

Recently, two randomized controlled trials investigated the addition of LMWH in the standard treatment regimen of COPD patients through periods of symptom exacerbation (Shi and Li, 2013; Qian *et al.*, 2014). In the study by Qian et al, the patient group treated with LMWH progressed with reduced mechanical ventilation duration (6.6 vs. 3.8 days; $p<0.01$), shorter ICU length of stay (8.5 vs. 5.6 days; $p<0.01$) and duration of hospitalization (14.3 vs. 11.3 days; $p<0.01$) (Qian *et al.*, 2014). Shi and Li demonstrated that patients subjected to LMWH demonstrated significantly improved pulmonary function test parameters, i.e. FEV1, FEV1/FVC and arterial blood gas indicators, i.e. SaO2, PaO2, PaCO2, when compared with the control group ($p<0.01$) (Shi and Li, 2013).

Brown et al additionally randomly analyzed patients with stable COPD, adding subcutaneously administered enoxaparin to standard inhaled salmeterol and fluticasone propionate treatment in 46 patients. Patients in the control group displayed a significant FEV1 increase only following 12 weeks (0.145 L, 95 % CI 0.994–1.406, $p<0.01$), patients additionally treated with enoxaparin,

however, demonstrated an FEV1 increase in all evaluations, reaching a peak of 0.244 L at the 12th week (95 % CI 1.175–1.596, p<0.01) (Brown *et al.*, 2006).

2.3. Heparin and post – thrombotic syndrome

Post – thrombotic syndrome (PTS) represents the most common complication after deep vein thrombosis, potentially affecting 20 to 50 % patients within two years following the acute occurrence. It is a condition characterized by chronicity and high prevalence which, apart from being an economic load to society, also negatively influences patients' quality of life (Kahn, 2006; Roberts *et al.*, 2014).

Even though the pathophysiology is not yet fully understood, it has been associated with persistent venous obstruction and occurrence of reflux. This hemodynamic situation's modification induces a state of venous hypertension, which is recognized as the factor that sparks all the related clinical manifestations. Of note, venous hypertension induces retention of leukocytes within the vascular bed, propagating numerous inflammatory mechanisms.

Yet again, the common denominator for the onset of the disease appears to be inflammation.

Anticoagulant drugs, heparins especially, are known to repress the embolization and growth of the thrombus, despite not inducing complete thrombolysis. For the deduction of severe forms of PTS, elastic compression stockings are often recommended, their adherence however in patients' everyday life remains limited. Several relative reviews have been published with controversial conclusions, as such not providing evidence of a clear benefit.

Chemical thrombolysis or mechanical interventions have an unclear effect on PTS reduction, albeit producing appealing results obtained throughout acute phase treatment. Their conflicting results as well as their high cost merit further research.

The pathogenetic processes would suggest that a potent anticoagulant treatment for acute VTE may repress PTS, disrupting valve damage and inflammatory process expression.

In a multicenter, randomized controlled trial (RCT) including 480 patients with VTE, the administration of tinzaparin (for 5 days) plus oral warfarin for 12 weeks (usual care) versus subcutaneous tinzaparin (175 IU/kg, 19/day for 12 weeks) was evaluated (Hull *et al.*, 2009). No difference was observed in recurrent rates of VTE between the two groups, i.e. at 12 weeks: 3.3 % in both groups and at 1 year: 10.4 % for tinzaparin vs. 8.3 % for usual care; no difference was also noted in bleeding and death rates. Patient satisfaction was increased in the tinzaparin group ($p = 0.0024$), even more so concerning the independence from blood monitoring. The same group reported lower leg ulcer incidence at 12 weeks ($p = 0.02$) and were further less likely to report PTS symptomatology ($p = 0.001$).

Numerous studies have shown that LMWH, when administrated for an extended duration, might augment thrombi recanalization. Residual venous thrombi are recongnized as a risk factor for recurrent thrombosis and PTS episodes and are indicators an underlying thrombophilic state (González-Fajardo *et al.*, 2008).

In a meta – analysis examining the efficiency of UFH against LMWH during acute VTE treatment (Hull, Liang and Townshend, 2011), LMWH was found to lead to reduction of thrombi size and recurrence risk.

2.4. Heparin and the pregnancy – parturition period

In women with VTE during pregnancy or childbirth, LMWHs are favored as the drugs of choice, mainly thanks to their not crossing the placental barrier and their safety towards the fetus. Even though LMWH dosing in non – pregnant females is facile, in pregnancy it is often complicated by weight gain and alterations in the glomerular filtration rate, particularly in the third trimester. In that event, dosage may be streamlined by monitoring anti – Xa activity levels.

As far as delivery is concerned, no consensus has been reached so far favoring on particular method. Clinical experience, on the other hand, dictates avoidance of epidural anesthesia in case of heparin therapeutic dose administration in the past 24 hours. In an ideal scenario,

heparin discontinuation is recommended the previous day before parturition, be that conducted either through normal delivery or caesarian section. In patients treated with LMWH once daily, a 50% dose reduction in the morning previous to the day of delivery is recommended.

Romualdi et al. published a systematic review and meta – analysis, evaluating bleeding complication risk and recurrence of VTE in patients developing VTE through the course of pregnancy treated with antithrombotic therapy (Romualdi *et al.*, 2013). Eighteen studies were reviewed, including 981 pregnant patients, of which 159 were treated with UFH and 822 with LMWH. Antigoagulant therapy was linked with a 1.41% weight mean incidence of major bleeding (95 % CI 0.60–2.41 %) before delivery and 1.90 % (95 % CI 0.80–3.60 %) in the following 24 h. The calculated weight mean incidence of VTE recurrence during pregnancy was 1.97 % (95 % CI 0.88–3.49 %). The authors reached to the conclusion that anticoagulant therapy seems safe an efficient for pregnancy – related VTE treatment, the optimal dosage regimens however remain inconclusive.

Another, more recent meta – analysis, comprising randomized trials comparing one method of thromboprophylaxis against either placebo or no treatment, or two (or more) methods against each other (Bain *et al.*, 2014), included pregnant females or females having delivered in the past 6 weeks, in increased VTE risk, reaching a total of 2592 included females. Concerning prepartum prophylaxis, either LMWH versus UFH or LMWH versus placebo did not detect any differences in symptomatic thromboembolic event, pulmonary embolism or symptomatic deep vein thrombosis occurrence. LMWH was associated with less discontinuation of therapy due to adverse event incidence (RR: 0.07; 95 % CI 0.01–0.54) and fewer fetal losses (RR: 0.47; 95 % CI 0.23–0.95) in comparison with UFH. In postpartum prophylaxis analysis, no differences were observed regarding the aforementioned parameters and major bleeding incidence. The conclusion made by the authors was that the evidence supporting recommendations for thromboprophylaxis in pregnancy and the early postpartum period still remains insufficient.

Identifying women at high risk for VTE is well – established; administering primary prophylaxis in asymptomatic thrombophilic patients, however, remains a question; anticoagulant treatment

in females with unexplained recurrent abortion history is a highly controversial practice, which may be on one hand increasingly usual, it is on the other hand still characterized by many inconsistencies.

On the event of abortion occurrence in the context of either diagnosed thrombophilia or complicated high – risk pregnancy, primary prevention through anticoagulant agents may constitute a valid option, still meriting however further research. Regarding primary prophylaxis, secondary prophylaxis and acute VTE treatment in all stages of pregnancy, LMWHs are the favored agent of choice, regardless of the indication (Middeldorp, 2013).

2.5. Anticoagulant treatment and cancer

Cancer and thrombosis have been interlinked since the nineteenth century. Trousseau described VTE as a neoplasm complication in 1865 and in 1878, Billroth detected the presence of malignant cell inside a thrombus, reporting therefore on cancer spread through thromboembolism (Falanga, 2004).

Data pooled in an analysis of 38 study populations led to the estimation of the annual VTE incidence among cancer patients ranging between 0.5% to 20%, depending on the type of tumor, its stage and the treatment selected, i.e. surgery, chemotherapy and radiotherapy (Horsted, West and Grainge, 2012). Pancreatic and brain tumors were recognized as the highest – ranking regarding VTE incidence. VTE risk is overall four times greater among cancer patients when compared with general population.

2.5.1. The antineoplastic properties and molecular mechanisms of heparins from preclinical studies

Frequent heparin administration for thromboprophylaxis produced evidence supporting a beneficial effect on the treatment of the disease through processes other than anticoagulation.

The role of platelet and leukocyte activation is an established factor in the mechanism of metastatic spread. The impact of heparin in metastasis decrease was shown in animal models. This activity is believed to be associated with the hindrance of fibrin deposits around tumor cells, disrupting their immune system response. The exact mechanisms, however, are not fully understood.

Numerous studies have demonstrated that heparin compounds with minimal anticoagulant property also prevent metastatic growth, marking the pleiotropic role of this medication. This activity probably derives from the suppression of heparinase function, expression of selectin and tissue factor pathway, all of which are related to anti – inflammatory function. Tissue factor (TF) is known to play a role in angiogenesis promotion and tumor growth. Heparin is believed to stimulate TF inhibitor release by EC, preventing tumor growth (Mandalà, Falanga and Roila, 2011; Khorana, 2012; Lee, 2012).

Even though the epidemiologic association between cancer and thrombosis has been already established, the pathophysiological processes intertwining tumor development and the hemostatic system are complicated and many aspects are not yet elucidated. The inactivation of particular suppressor genes, such as p53 and PTEN, or the activation of specific oncogenes, e.g. MET, EGFR and K-ras, promote TF, plasminogen activator inhibitor 1 (PAI-1) and COX-2 overexpression in tumor cells, indicating that the activation of the hemostatic system partakes in a genetic program reinforces the transformation and progression of tumors (Boccaccio and Comoglio, 2009; Kuderer, Ortel and Francis, 2009). Tumor cells stimulate coagulation by generating a pro-coagulant environment surrounding the tumor, heightening thrombotic risks. Additionally, the activated hemostatic system modifies cell proliferation and survival, tumor angiogenesis, invasion and dissemination, as well as formation of metastases (Smorenburg and Van Noorden, 2001), forming therefore a vicious cycle.

Coagulation is directly activated by tumor cells through expression of pro-coagulants on cell surfaces or through their secretion into the extracellular environment as TF (Prandoni, Falanga and Piccioli, 2005), as cancer pro-coagulant (CP) (the abbreviation will not be applied to avoid confusion with the abbreviation for the Classical Pathway of coagulation) (Falanga and Gordon,

1985) and, to a lower extent, tumor mucins (Varki, 2007); coagulation is also indirectly activated by tumor cells through adhesion molecule expression which activate immune system cells, specifically macrophages and neutrophils, as well as platelets or through release of cytokines, i.e. interleukin 1 (IL-1), growth factors (VEGF) and tumor necrosis factor (TNF) (Kuderer, Ortel and Francis, 2009). Similarly, tumor cells engage in an interaction with a wide range of cells, e.g. immune cells like monocytes and macrophages, platelets, ECs, propagating thrombosis by platelet activation and coagulation stimulation, but also by supporting tumor cell invasion, extravasation and dissemination (Rickles and Falanga, 2001). Cellular interactions take place directly between adhesion proteins, as for example E-Selectins and P- and L- integrins, vascular adhesion molecule 1 (VCAM1), carcinoma mucins and endothelial receptors GPIIIa and GPIIb on tumor cell and healthy cell surfaces. Cellular interactions further occur indirectly via cytokine release, as for example IL-1, IL-6, VEGF and TNF-α (Bendas and Borsig, 2012).

Taking those interactions into account, the hemostatic system's role on tumor genesis and progression is pivotal to comprehend, since an increasing body of evidence has linked it with cell proliferation and survival, tumor angiogenesis, invasion, dissemination and metastasis formation. In the context of said activity, numerous factors are especially relevant: firstly, thrombin, TF and protease – activated receptors (PARs) are important in proliferative, apoptotic, and pro-angiogenic programs. Secondly, the fibrin matrix is integral in the course of tumor growth and the process of metastasis. Thirdly, selectins are necessary for metastasis development (Borsig *et al.*, 2001, 2002; Rickles, Patierno and Fernandez, 2003; Prandoni, Falanga and Piccioli, 2005; Borensztajn and Spek, 2008; Boccaccio and Comoglio, 2009; Borsig, 2010).

The mechanisms that associate tumor biology and thrombin have sparked a hypothesis that preventing coagulation with anticoagulant treatment might have an antitumor function beyond their established antithrombotic activity. An antineoplastic action which is associated with anticoagulant activity is employed by Vitamin K antagonists (VKA). LMWH also demonstrate antineoplastic effect regardless of anti – Factor Xa and IIa anticoagulant activity. Several animal studies have confirmed a survival prolongation with heparin after inoculation of tumor cells.

The antineoplastic functions of heparin collect numerous proposed mechanisms of action, which have been indicated based on a number of studies (Hejna, Raderer and Zielinski, 1999; Smorenburg and Van Noorden, 2001), addressing particularly the proliferation (Au *et al.*, 1993), adhesion and migration mechanisms, necessary for metastasis (Amirkhosravi *et al.*, 2003; Stevenson, Choi and Varki, 2005) and also, angiogenesis (Khorana *et al.*, 2003; Mousa and S Mohamed, 2004; Mousa and Seema Mohamed, 2004; Mousa *et al.*, 2004). By binding to mac 25, also referred to as tumor – adhesion factor (TAF), high heparin concentrations are able to hinder endothelial cell tubular structure formation, indicating its important role in the primary steps of angiogenesis. On the other hand, LMWH and tinzaparin particularly, shortens endothelial proliferation in vitro in a dose – dependent manner. The inhibition of alternative pro – angiogenic functions exerted by heparin sulfate proteoglycans, protease-activated receptor 2, or by hindering hepatocyte growth factor or dispersion factor might constitute an opportunity to pair heparin with antiangiogenic drugs. Heparin has further been shown to prevent the cellular uptake of extracellular vesicles, producing an alternative antitumor process by inhibiting neovascularization. A suppressing effect has also been demonstrated among extracellular binding proteins, which is essential for GM cell migration and survival, although the relative studies demonstrate controversial findings (Au *et al.*, 1993; Jayson and Gallagher, 1997; Li *et al.*, 2001; Amirkhosravi *et al.*, 2003; Khorana *et al.*, 2003; Mousa and S Mohamed, 2004; Mousa and Seema Mohamed, 2004; Mousa *et al.*, 2004).

2.5.2. Cancer – associated thrombosis

Cancer – associated thrombosis is a noteworthy source of mortality and morbidity. Following Trousseau's observation mentioned in the beginning of the present subject, knowledge regarding this pathophysiology has grown leaps and bounds. TF is a pivotal trigger of cancer – associated thrombosis. In the extrinsic pathway of coagulation, the transformation of factor X to its active form is catalyzed by TF. Several tumors express TF (Callander, Varki and Vijaya Rao, 1992) and high TF levels increase thrombosis risks in the context of advanced cancer (Zwicker *et al.*, 2009). In a clinical trial of patients with cancer, with increased levels of circulating TF – bearing microparticles (Zwicker *et al.*, 2013), the highest risk of thrombosis was evident for the individuals with the most increased levels. These patients seemed to benefit the most from

heparin anticoagulation, demonstrating a 5.6% rate of VTE upon enoxaparin administration, significantly lower than the 27.3% rate of VTE in the placebo arm.

There are two primary mechanisms with which heparin interacts with the TF pathways. Firstly, heparins directly lower TF expression and function, by altering vascular growth and endothelial factors (Ettelaie *et al.*, 2011). Via the interruption of these mediators' activity, LMWH reduces the transcriptional activity of NF – kappaB. Secondly, heparin interacts with tissue factor pathway inhibitor (TFPI), one of the major constraints of the procoagulant activity of the activated TF/factor VIIa complex. EC exposure to either UFH or LMWH instantly releases TFPI from the cell surface and ultimately propagates a sustained augmentation of TFPI production and excretion (Lupu *et al.*, 1999). The heparins therefore, by hindering TF via these two mechanisms, may be especially efficient in cancer – related VTE treatment.

Besides TF, alternative mediators are linked with cancer – related thrombosis. Neutrophil priming has been evident in mice with early stage tumors. During tumor progression, neutrophil activation and neutrophil extracellular trap (NET) formation (discussed in detail below) take place simultaneously with venous thrombosis (Demers *et al.*, 2012). Patients subjected to chemotherapy are at an increased risk for thrombosis and NET formation may be the culprit of this association (Van Den Berg and Reitsma, 2011). Heparin inhibits NET – induced thrombosis through the removal of platelets and the interaction with the histones on NET filaments (Fuchs *et al.*, 2010).

In a landmark study published in 2003, the CLOT study (Lee *et al.*, 2003), cancer patients with acute VTE treated with LMWH monotherapy demonstrated a nearly 50% reduced recurrence rate of VTE in comparison with those treated with LMWH "bridging" therapy to warfarin. Both LMWH and UFH augment TFPI which inactivates and increases the TF – factor VIIa complex clearance (Lupu *et al.*, 1999). These functions may explain heparin's superiority over warfarin in the treatment of malignancy – associated thrombosis.

The rising use of direct oral anticoagulants has sparked a hypothesis that said agents could substitute heparin in the treatment of cancer – associated thrombosis. However, heparins, besides exerting anti – Xa action, further affect additional pathways, such as TF, TFPI and NETs.

As such, they may prove superior in the treatment of cancer – associated thrombosis when compared against direct oral anticoagulants. Edoxaban was recently found non – inferior to LMWH in terms of recurrent VTE or major bleeding (Raskob *et al.*, 2018), i.e. lowered the rate of recurrent VTE (difference in risk, -3.4 percentage points; 95% CI, -7.0 to 0.2) but increased the rate of major bleeding (difference in risk, 2.9 percentage points; 95% CI, 0.1 to 5.6). Finally, a clinical trial is currently ongoing, evaluating LMWH versus the direct anticoagulant apixaban for the treatment of malignancy – associated acute VTE (ClinicalTrials.gov identifier NCT02585713).

2.5.3. Anticoagulant therapy's impact on the survival of cancer patients

As soon as the early 1980s, RCTs have been conducted which investigated the impact of anticoagulants on cancer patients' survival; the first to be tested were warfarin and vitamin K antagonists and later on, UFH and LMWH. Both RCT and early meta-analyses results were controversial (Zacharski *et al.*, 1981; Lebeau *et al.*, 1994; Hettiarachchi *et al.*, 1999; Smorenburg *et al.*, 1999, 2001; Haas *et al.*, 2012).

A recent systematic review and meta-analysis examined the efficiency and safety of adjunctive coagulation in lung cancer patients with no indication for anticoagulant treatment (Zhang *et al.*, 2013). 2185 patients enrolled in 9 studies were evaluated for one-year survival rates and VTE incidence. The results demonstrated that anticoagulation had no effect on the six-month survival, it did however significantly improve one-year (RR 1.18,95 % CI 1.06–1.32; p = 0.004) and two-year (RR 1.27, 95 % CI 1.04–1.56; p = 0.02) survival rates. Of note, the benefit was evident for patients with small-cell lung carcinoma (SCLC) and those without advanced tumors.

On the contrary, different results were describes in another meta-analysis published that year (Che *et al.*, 2013). 11 RCTs with 7284 patients (3835 cases and 3449 controls) were analyzed to estimate one-year mortality rates, thromboembolism occurrence and adverse bleeding in cancer patients without evidence of VTE treated with LMWH. In contrast with Zhang et al, patients' diagnoses included a wide range of tumors, i.e. either small or non-SCLCs, pancreatic,

prostate, breast and ovarian tumors of variable staging, also treated with a variety of medications, i.e. enoxaparin, dalteparin, nadroparin, certoparin and semuloparin with different treatment durations and regimens, ranging from six weeks to one year. The results demonstrated significant difference in both adverse bleeding risk (relative risk of 1.32, 95 % CI 1.08–1.62) and VTE occurrence (RR: 0.53, 95 % CI 0.42–0.67) on comparison of LMWH versus placebo or no anticoagulant. The authors did not observe any significant difference in neither one-year mortality rate (RR: 0.97, 95 % CI 0.92–1.02) nor major bleeding incidence (RR: 1.22, 95 % CI 0.87–1.71).

A great number of RCTs have been conducted among particular cancer populations. An RCT including 38 patients with a recent diagnosis of limited-stage SCLC compared chemo- and radiation therapy with or without bemiparin 3500 IU per day for a duration of up to 26 weeks (Lecumberri *et al.*, 2013). The slow recruitment rate led to an early termination of the study, which demonstrated however that both median overall survival (OS: 1133 vs. 345 days, HR 2.96, 95 % CI 1.22–7.21; p = 0.017) and median progression-free survival (PFS: 410 vs. 272 days, hazard ratio [HR] 2.58, 95 % CI 1.15–5.80; p = 0.022) were significantly increased in the patient group treated with bemiparin.

In another study, 503 patients with a locally advanced pancreatic cancer or hormone-refractory prostate cancer or non-SCLC of IIIB staging were randomly allocated to anti – cancer therapy (van Doormaal *et al.*, 2011) with or without subcutaneous nadroparin (for a 6-week duration, eligible for additional cycles). Heparin treatment lasted for a mean duration of 12.6 weeks. The overall mortality demonstrated neither significant nor clinically relevant difference (56.6 % in the nadroparin group vs. 61.8 % in the control group, adjusted HR 0.94; 95 % CI 0.75–1.18, p = 0.565); so did the median survival (13.1 vs. 11.9 months; adjusted HR 1.03, 95 % CI 0.81–1.30, p = 0.819), progression-free survival (5 vs. 5.8 months), major bleeding (4.1 % in the treated group vs. 3.5 % in the control group), clinically relevant bleeding (9.4 vs. 8.1 %; p = 0.638) and thromboembolic events.

So far, the body of evidence being reviewed is not capable of concretely establishing the real effect of heparin on cancer patient survival, which medications and treatment modalities are

efficient and more importantly, among which patient groups is administration recommended.

Since a great number of studies have been conducted, employing different anticoagulant treatments in patients with different cancer types, a reference to the survival benefit of each specific anticoagulant medication seems plausible.

a. VKA treatment's effect on survival

VKA treatment was the first anticoagulant medication to be related with the association of anticoagulant drugs and cancer development. A study published in 2001 (Schulman and Lindmarker, 2000) reported a reduced cancer incidence of diagnoses of cancer among patients with thrombosis treated with VKA for a duration of six months, compared with those treated for six weeks (incidence ratio 3.4, 95% CI 2.2–4.6), putting into doubt the observations of preceding studies which had failed to demonstrate an overall effect of said drugs on mortality rates. Five either randomized or cohort studies have been conducted putting this issue under scrutiny (Zacharski *et al.*, 1981; Chahinian *et al.*, 1989; Daly, 1991; Levine *et al.*, 1994; Maurer *et al.*, 1997). Smorenburg et al collected these data and their pooled analysis was published in a systematic review in 2001 (Smorenburg *et al.*, 2001). The primary outcome was that overall one – year mortality in patients with cancer was not altered by treatment with VKA, with a 0.89 odds ratio (OR) (95% CI 0.70–1.13). In spite of its limitations and general results, this study suggested a hypothesis that patient subgroup with small – cell lung carcinoma (SCLC) might actually show a survival benefit.

b. Unfractionated heparin's effect on survival

An interesting observation was that these results were fairly consistent with these presented by the same author concerning UFH's potential antineoplasmatic effect. A number of RCTs (Papaioannou *et al.*, 1986; Fielding *et al.*, 1992; Lebeau *et al.*, 1994; Nitti *et al.*, 1997) and non – randomized studies (Kohanna *et al.*, 1983; Törngren and Rieger, 1983; Kingston, Fielding and

Palmer, 1993; Kakkar *et al.*, 1995) were included in a systematic review (Smorenburg *et al.*, 1999) published in 1999. In a subgroup analysis conducted by the authors, patients receiving prophylactic doses were compared against patients receiving therapeutic doses of UFH. This study failed to present a net impact of UFH on overall survival. The authors observed, in the subgroup of RCTs, an increased three – year mortality rate among patients with gastrointestinal cancer, who were administered prophylactic UFH (Fielding *et al.*, 1992; Levine *et al.*, 1994; Nitti *et al.*, 1997); on the contrary, in a study assessing the effect of UFH among patients with microcytic lung cancer (Lebeau *et al.*, 1994), an improved survival rate was demonstrated, although it failed to reach statistically significant levels [OR 0.64, 95% (CI) 0.25–1.62].

c. Low molecular weight heparin's effect on survival

Responding to two RCTs comparing LMWH – treated and UFH – treated patients (Green *et al.*, 1992; Prandoni *et al.*, 1992) which demonstrated a difference in unexpected death not attributed to the occurrence of hemorrhage and re-thrombosis, a meta – analysis (Siragusa *et al.*, 1996), which included RCTs performed from 1980 to 1994, displayed a relative risk (RR) of 0.51 for overall mortality in favor of the LMWH – treated group (95% CI 0.2–0.9, p = 0.01). A second analysis (Hettiarachchi *et al.*, 1999) including work performed until 1997, produced similar results. The authors reported an OR of 0.61 for three – month mortality (95% CI 0.40–0.93) in favor of LMWH. Additionally, in this analysis, it was confirmed that this lowered risk was accounted for through the difference in mortality attributed to hemorrhage and re-thrombosis. These results, despite having been obtained from retrospective studies, indicated that LMWH may possess antitumor functions and rejuvenated the hypothesis that anticoagulant medications may display antitumor capacity.

Two additional meta – analyses followed later on. The first one (Lazo-Langner *et al.*, 2007) analyzed four published studies; two were open – label, whereas the other two were randomized, double – blinded and placebo – controlled. The primary outcome was the survival in all of them. Of note, there was heterogeneity in regard to patient characteristics and treatment (Altinbas *et al.*, 2004; Kakkar *et al.*, 2004; Klerk *et al.*, 2005; Sideras *et al.*, 2006).

At one year, an absolute risk (AR) in risk of death of 0.70 was noted (95% CI 0.49–1.00, p = 0.05) and also a relative reduction in the one – year risk of mortality of 0.87 (95% CI 0.77–0.94, p = 0.04); both in favor of the experimental group. At two years, an AR of 0.57 (95% CI 0.34–0.96, p = 0.04) and a relative reduction in one – year risk of mortality of 0.89 (95% CI 0.80–0.99, p = 0.03) were observed. Albeit by a narrow margin, these observations confirmed the beneficial impact of nadroparin and dalteparin on overall survival (OS). Taking into account that a more potent effect on early disease stages has been hypothesized, a study by Altinbas et al was designed, excluding patients in stages I and II; the benefit in regard to AR and RR remained, however. The aforementioned results, further than reaching conclusions concerning the effect on survival, verified the safety of LMWH in patients with advanced cancer with no previous thrombosis.

Upon separate examination of these four studies' results, two have exhibited a significant survival difference. Altinbas et al noted the greatest benefit (Altinbas *et al.*, 2004), having randomized 84 patients with SCLC to receive the standard treatment, i.e. epirubicin, cyclophosphamide and vincristine in 6- and 21-day cycles) or standard treatment with dalteparin in 5000 i.u. per day regimen for a duration of the 18 weeks of treatment. It was shown that a good status was mostly male, presenting with both limited (n=36) and advanced disease (n=48). The experimental group demonstrated an increased rate of response in comparison with the control group (69.2 vs. 42.5% p = 0.07). The median OS was 13.0 versus 8.0 months (p = 0.01), while the median progression – free survival (PFS) was 10.0 versus 6.0 months (p = 0.01). A reduction in the risk of mortality of 0.56 was also noted (95% CI 0.30–0.86, p = 0.012). No difference was observed among the two groups in regard to disease staging and toxicity. In another study (Klerk *et al.*, 2005), 302 patients with tumors of variable localization and histology which were either advanced locally or metastatic were randomized to receive six weeks of the standard treatment with or without nadroparin adjusted for weight. The study produced positive findings, the six – month OS was 61 vs. 56%, while at one year it was 39 vs. 27% and at two years 21 vs. 11%; the RR of death was 0.75 (95% CI 0.59–0.96). In a subgroup analysis planned beforehand, an increased RR and greater OS were noted in the patients who had a life expectancy of more than six months compared to those with a shorter life

expectancy, corroborating the postulation that, patients with a better prognosis benefit the most from heparin's impact. Those patients demonstrated a 0.64 RR of mortality (95% CI 0.45–0.90) and a median survival of 15.4 months versus a 0.88 RR of death (95% CI 0.62–1.25) and a median survival of 9.4 months, observed in the arm with a graver prognosis. Again, no difference in terms of adverse bleeding events was noted.

The remaining two studies, though, presented conflicting results. In the FAMOUS study (Kakkar *et al.*, 2004), the superiority of dalteparin was demonstrated, when added to the standard treatment regimen alone in patients with breast, digestive tract, genitourinary tract and gynecological carcinomas. The majority of those exhibited locally advanced or metastatic disease; no significant difference was noted in bleeding event incidence. The one – year OS was 46% (95% CI 39–53) in the experimental arm and 41% (95% CI 34–49) in the control arm. The survival was, at two years, 27% (95% CI 20–34) versus 18% (95% CI 11–25) and at three years, 21% (95% CI 14–28) versus 12% (95% CI 5–19) for the experimental and the control group, respectively. Despite it being a negative study, a benefit in survival was again noted in the preplanned subgroup analysis, which consisted of patients with survival beyond 17 months. The patients in the experimental arm of this subgroup had a 78% survival rate at two years and 60% at three years, versus the respective survival rates of the placebo group, 55% and 36% (p=0.03), whereas the mean survival times 43.5 months (95% CI 33 – 52.3 months) versus 24.3 months (95% CI 22.4–41.5 months). Again, following this sub-analysis, the hypothesis that patients with a more favorable prognosis would benefit more from the addition of heparin to the standard treatment was further supported. A randomized, double – blinded studied, initially placebo – controlled trial studied the use of LMWH in a sample of 138 patients with lung, breast, colon and prostate cancer, also locally advanced or metastatic disease, PS 0-2, being nursed after first – line treatment failure (Sideras *et al.*, 2006). The study's design was modified due to the slow recruitment rate and the placebo arm was deleted, therefore patients received standard clinical treatment with or without LMWH. The primary outcome was OS and no significant difference was noted among the combined LMWH arms and the combined standard care and placebo groups.

A meta – analysis in 2014 (Sanford *et al.*, 2014) drew the opposite results from the preceding one (Lazo-Langner *et al.*, 2007). Five novel studies were included (Agnelli *et al.*, 2009, 2012; Perry *et al.*, 2010; van Doormaal *et al.*, 2011; Lecumberri *et al.*, 2013), with a total of 5098 subjects, the majority of whom presented with locally advanced or metastatic disease. Considerable heterogeneity was observed in regard to oncological disease and intervention. All were RCTs which compared LMWH with placebo or no anticoagulant medication with a 0.87 OR for one – year mortality (95% CI 0.70–1.08, p = 0.21) and an overall RR for one – year mortality of 0.94 (95% CI 0.86–1.04, p = 0.24). A significant reduction was detected in the thrombotic event risk, with an RR of 0.59 (95% CI 0.42–0.83, p = 0.002); no significant increase was observed in the bleeding risk of the patient group subjected to heparin administration.

Upon examination of the five added studies included in the second meta – analysis, the two studies by Agnelli et al were those contributing the greatest number of patients and they were both negative. In the former one (Agnelli *et al.*, 2009), survival was established as a secondary outcome, while the primary outcome was arterial or venous thrombotic event incidence, found significantly reduced in the experimental arm. Following one year past randomization, in the nadroparin – receiving group, mortality was 43.3% and 40.7% in the control arm, failing to reach statistically significant levels. The latter study also evaluated thrombotic event incidence as a primary outcome (Agnelli *et al.*, 2012). Similarly, the patient sample consisted of patients with variable solid tumors and no previous thrombosis. In this study, the heparin added to the standard treatment was semuloparin, an ultra – LMWH. The groups demonstrated no significant difference in thrombotic events. In the experimental group, the mortality rate was 43.4% versus 44.5% in the placebo group (hazards ratio 0.96, 95% CI 0.86–1.06, p = 0.40).

Another study (van Doormaal *et al.*, 2011) also failed to exhibit an effect on survival. In this study, 503 patients with locally advanced or metastatic cancer of the pancreas, lung and prostate were randomized to receive standard treatment with or without nadroparin for six weeks. The mortality was the primary outcome and time – to – progression was a secondary outcome. In neither of those variables was a significant difference observed, with the overall

mortality reaching a rate of 56.6% versus 61.8%. In the nadroparin – receiving group, the median survival was 13.1 months and 11.9 months in the control arm.

The primary outcome of the PRODIGE trial was to reduce the thrombotic event incidence and included 186 patients with glioma (Perry *et al.*, 2010). Since the study medication was withdrawn, the study had to be terminated prematurely. Neither a thrombotic event reduction nor a survival benefit was demonstrated; instead, the experimental group had a higher mortality rate. This predicament along with an increased incidence of adverse bleeding, albeit insignificant, led to reluctance towards the prophylactic administration of heparin in brain tumors.

Another RCT, on the other hand, the ABEL trial generated positive results, associating LMWH with increased survival in cancer patients (Lecumberri *et al.*, 2013). The enrolled patients presented with SCLC of limited stage and the primary endpoint was survival with the disease in remission. A slow recruitment rate led to the study being ended prematurely, a point at which only 38 patients had been included, contributing but a small number of patients to the total meta – analysis. With the standard treatment, PFS was 272 days, whilst with bemiparin added to the standard treatment, it was 410 days; the hazard ratio (HR) was 2.58 (95% CI 1.15–7.21, p = 0.022) and the OS was 345 vs. 1133 days (95% CI 1.22–7.21, p = 0.0017). No significant difference was noted in either bleeding or response rate. As such, the two meta – analyses that present the most crucial evidence, reached conflicting conclusions. The limitations in the first meta – analysis were the small size of the population, a lack of statistical power and the heterogeneous results among the different authors. The second meta – analysis, despite including a higher number of subjects with a relative weight specifically higher than the first three studies (Agnelli *et al.*, 2009, 2012; van Doormaal *et al.*, 2011), failed to demonstrate a significant increase or a trend towards greater survival. Surprisingly, out of the three studies exhibiting positive results included in the two meta – analyses (Altinbas *et al.*, 2004; Klerk *et al.*, 2005; Lecumberri *et al.*, 2013), only the two included subjects with SCLC and applied the standard treatment as the conventional group (Altinbas *et al.*, 2004; Lecumberri *et al.*, 2013).

These remarks stimulated the hypothesis that the design of the study is a crucial factor in assessing for discrepancies on the anticoagulants' effect on survival.

Four RCTs have more recently assessed the effect of LMWH on the survival of patients with several cancer types (Pelzer *et al.*, 2015; Macbeth *et al.*, 2016; Ek *et al.*, 2018; Meyer *et al.*, 2018). In the CONKO-004 trial, first – line chemotherapy with or without enoxaparin were compared in terms of survival and VTE incidence, in a population of 312 subjects with pancreatic cancer of advanced staging (Pelzer *et al.*, 2015). Enoxaparin was found to lower VTE incidence, there were no differences, however, either in the PFS (HR 1.06, 95% CI 0.84–1.32, p = 0.64) or the OS of the two groups (HR 1.01, 95% CI 0.87–1.38, p = 0.44). The FRAGMATIC study (Macbeth *et al.*, 2016) included 2022 individuals with a recent pulmonary cancer diagnosis of any staging and any histopathology, randomized to receive the standard treatment with or without LMWH prophylaxis for a duration of 24 weeks. The trial did not reach the number of events aimed for the primary analysis, but there was no significant difference among the trial groups in terms of OS (1.01, 95% CI 0.93–1.10, p = 0.814). The RASTEN study (Ek *et al.*, 2018) was performed among individuals with recently diagnosed SCLC, in whom the standard treatment was provided with the addition of enoxaparin administered at supraprophylactic doses, found to have an effect neither on their PFS (HR 1.18, 95% CI 0.95–1.46, p = 0.14) nor their OS (HR 1.11, 95% CI 0.89–1.38, p = 0.36). Finally, in an RCT (Meyer *et al.*, 2018) performed in 549 patients with non – metastatic resected non – SCLC of stages I, II or IIIA, with a median follow – up of 5.7 years, the standard treatment with added tinzaparin at 100 i.u./kg once daily for 12 weeks displayed no significant benefit on OS, when compared with the standard treatment alone (HR 1.24, 95% CI 0.92–1.68, p = 0.17).

d. <u>Low molecular weight heparin's effect on survival in patients with brain or other location tumors</u>

Taking into account the peculiar characteristics of brain tumors, special consideration is appropriate in this context. The most common primary tumor is Glioblastoma Multiforme

(GM), characterized by a grave prognosis. Despite progressive developments, the need for more satisfactory treatment modalities is dire (Akaogi *et al.*, 1996).

Although the evidence is limited, a number of studies indicate that heparin might alter the progression of GM (Akaogi *et al.*, 1996; Lund *et al.*, 2003; Zhao *et al.*, 2010; Svensson *et al.*, 2011; Christianson, van Kuppevelt and Belting, 2012). The suppressing impact of LMWH on the growth of said neoplasms has been demonstrated in some preclinical trials, which might implicate angiogenesis, a hallmark of GM. Through binding with mac 25, commonly known as tumor – derived adhesion factor (TAF), heparin in great concentrations is able to hinder endothelial cell tubular structure formation, indicating its important role in the initial steps of angiogenesis. The mechanisms with which heparin may inhibit GM advancement are described in paragraph 2.5.1.

Three studies have assessed the impact of heparin and LMWH on GM patients' survival rates. The PRODIGE trial (Perry *et al.*, 2010), an RCT reviewed in the meta – analysis of 2014 (Sanford *et al.*, 2014) was the only study to draw negative results and was ended prematurely after the introduction of temozolamide in 2004. In another study, (Robins *et al.*, 2008), the enrolled patients were treated with radiotherapy and dalteparin prophylaxis; the primary outcome was OS. Administration of dalteparin could continue past progression. The control group included patients treated with radiotherapy in the past. The experimental group demonstrated a median survival of 11.9 months (95% CI 10–14), but no comparison was possible with the controls, as no such report was available; a trend towards improved OS was evident, but not enough to reach statistically significant levels ($p = 0.47$). Finally, another, retrospective study of small size (Zincircioglu *et al.*, 2012), including 30 patients subjected to surgical excision (radical or biopsy) of GM and following chemotherapy. Out of those patients, 13 received 4000 i.u. of enoxaparin daily, for 6 weeks. The group receiving enoxaparin displayed significantly increased one – year OS, i.e. 84.6% versus the control group's 41.2% ($p = 0.016$). The benefit seemed to remain in during the second year, albeit not achieving statistical significance. Nevertheless, this study had a limitation in the patients not being randomized to receive LMWH, because patient selection was conducted based on thromboembolism risk.

Although these results were promising, they have not led to the universalization of LMWH treatment even in the group selected based on their thrombotic risk and in the context of prophylaxis. The lack of a generalized LMWH treatment is not without merit; it is partially attributed to the risk of intracranial bleeding in GM patients, there are however, no appropriately designed clinical trials supporting its use.

2.5.4. Heparin pleiotropic effects among ambulatory patients

In the last 14 years, randomized studies have been conducted including a great number of patients that have note presented a benefit in survival for ambulatory LMWH prophylaxis. Aside from the PROTECHT40 study (Agnelli *et al.*, 2009), in the FRAGMEN-UK41 trial (Maraveyas *et al.*, 2012) consisted of a population of 123 individuals with pancreatic cancer. Following a similar pattern, the researchers compared gemcitabine with and without dalteparin prophylaxis. A reduction in VTE occurrence, the study's primary endpoint, was demonstrated in the experimental group receiving dalteparin (23 vs. 3.5%, p = 0.002); the survival rates did not exhibit any difference. Another study in 2015 (Pelzer *et al.*, 2015) involved a patient group with locally advanced or metastatic pancreatic cancer and high risk for thrombosis, reaching a total of 312 patients, who were randomized to receive chemotherapy, i.e. gemcitabine, cisplatin and 5 – fluoruracil, with or without 1mg/kg enoxaparin daily for three months, followed by 40 mg daily up to the point of disease progression. There was a significant three – month reduction in symptomatic VTE (HR 0.2, 95% CI 0.03–0.52, p = 0.001) with no difference in survival rates (HR 1.01, 95% CI 0.87–1.38, p = 0.44).

The randomized trials TOPIC – 1 (Robins *et al.*, 2008) and TOPIC – 2 (Haas *et al.*, 2012) assessed the addition of certoparin at 3000 i.u. per day for six months versus a placebo in terms of survival in patients with locally advanced or metastatic breast or lung cancer and reported no differences. In both RCTs, mortality rates were similar in both groups, also exhibiting the similar trend in the occurrence of asymptomatic or symptomatic thrombotic events.

Finally, there have been attempts to evaluate heparin's effects on survival of patients in the context of palliative care. In a study of 20 VTE patients (Weber *et al.*, 2008) nursed in the hospital with prognosis shorter than six months, randomized to receive subcutaneous nadroparin 2850/3,800 U (< 70/> 70 kg) or no treatment, no positive results were produced in regard to OS following three months of follow – up, with no significant differences between the two groups being reported.

2.5.5. Recent clinical trials

Several studies have just been published and others are currently ongoing, either in the recruitment phase or under analysis. Among them, a highlighted one was the NVALT – 8 study (Groen *et al.*, 2019), published in 2019, a randomized multicenter phase 3 study including 235 patients with fully resected non – SCLC, randomized following resection to receive either chemotherapy alone or with added nadroparin; the primary outcome was disease – free survival. The slow recruitment rate led to accrual being terminated sooner; the median disease – free survival was 65.2 months in the nadroparin – treated group and 37.7 months in the control group. No difference was reported in bleeding incidence among the two groups. Therefore, the authors reached to the conclusion that additional nadroparin did not improve disease – free survival in patients with non – SCLC subjected to surgery.

Another most recent noteworthy study, promoted by the Ottawa Hospital Research Institute, was the PERIOP – 01 (Auer *et al.*, 2022) published in January 2022. The trial was deigned to assess extended perio – operative administration of tinzaparin versus no anticoagulation in patients with resectable colorectal cancer. The primary outcome was once again disease – free survival (of three – year duration) with an experimental group being administered 4500 i.u. of tinzaparin per day for 56 days after the resection in comparison with a control group being treated with the usual prophylaxis. The recruitment was terminated prematurely on the grounds of futility following a previously defined interim analysis, after having recruited 614 patients out of the 1075 originally planned. The three – year disease – free survival was 78.9% in the tinzaparin arm versus 80.5% in the control arm (HR 1.09; [95% CI 0.91, 1.31; p=0.3]). The OS at five years was 91.3% in the experimental group and 92.4% in the control group (HR 1.08;

[95% CI 0.66, 1.79; p=0.1]). Adverse post – operative bleeding and VTE events demonstrated low occurrence rates. The authors concluded that tinzaparin as a part of extended – duration perioperative coagulation did not provide significant benefit in terms of either disease – free survival or OS among patients subjected to colorectal cancer resection.

2.6. Heparin and the glycocalyx

2.6.1. The endothelial glycocalyx

The interplay between proteoglycans, glycoseoaminoglycans (GAGs) and derivatives of membrane glycoprotein in the bloodstream is an important parameter to take into account in order to comprehend heparin's mode of action.

Membrane glycoproteins are expressed on the surface of all cells, attracting and aggregating GAGs and proteoglycans via the charge of cell surfaces and specifically on the circulating cells' surface, termed as glycocalyx (Nieuwdorp *et al.*, 2005).

At the EC level, the glycocalyx is an important sentinel contributing the majority of functions and particular characteristics attributed to the EC, but actually being corollaries of the glycocalyx.

As such, the EC may be known as the ideal antithrombotic cell, its activities however are thanks to the glycocalyx inhibiting direct interaction with the circulating cells, especially platelets and also thanks to the glycocalyx aggregating coagulation inhibitors, specifically TFPI, protease nexin and antithrombin, which hinder the coagulation factors' protease activity.

The EC is suggested to be essential in flow regulation, even more so in microvascular flow, something that explains flow – mediated vasodilation. For the synthesis of mediators by the EC, e.g. prostacyclin, endothelin, NO, etc., in order to regulate vascular motion, a sensor is needed on its luminal surface for this circulatory speed. This sensor is produced by the glycocalyx, which is to some extent induced by shear stress limitations created by blood flow velocity on

the EC's luminal surface, translating this mechanical force into an intracellular message through conformational alterations in transmembrane glycoproteins.

The glycocalyx exerts anti – inflammatory function, since it hinders the direct interaction with inflammatory cells: the circulating leukocytes' glycocalyces fend each other off with the endothelial glycocalyx. The latter also reduces the inflammatory cytokines' transendothelial passage.

The permeability for tissue exchange is regulated by the endothelium; this function is again a result of the activity of the glycocalyx, which is more permeable to water – soluble, rather than lipid – soluble molecules, as a consequence of its concentration of a high negative charge. As far as water – soluble molecules are concerned, their permeability is inversely proportional to the size: the larger the molecule, the more difficult their passing through the glycocalyx.

A great number of pathologies perturb the functionality of the glycocalyx and even though the endothelium may be still physically present, in case of its activation and/or in case of glycocalyx injury, its activities will be altered. The loss of either a part or a total of the glycocalyx leads to a reduction or absence of the aforementioned effects: anti – thrombotic, anti – inflammatory, flow – regulating and permeability actions.

Glycocalyx anomalies have been described in several pathologies (Tarbell and Cancel, 2016): in cancer and even more so in chemotherapy regimens, since these induce endothelial, or at the very least glycocalyx, destruction. Likewise, a pathological glycocalyx is also observed in diabetes; the glycocalyx is damaged by hyperglycaemic peaks, through release of measurable glycans into the bloodstream (Lemkes *et al.*, 2012). Glycocalyx anomalies have further been described in pregnancy, especially in cases implicating placental vascular complications and also in major burns, acute coronary syndromes, obesity and metabolic syndromes (Culty *et al.*, 1990; Rosenberg *et al.*, 1997; Henry and Duling, 1999; Conway, Collen and Carmeliet, 2001; van den Berg, Vink and Spaan, 2003; van Haaren *et al.*, 2003; Fransson *et al.*, 2004; Megens *et al.*, 2007; Pahakis *et al.*, 2007; Lee *et al.*, 2009; Woodcock and Woodcock, 2012; Henderson-Toth *et al.*, 2012; Kolářová *et al.*, 2014; Puchwein-Schwepcke *et al.*, 2021; Puchwein-Schwepcke, Genzel-Boroviczény and Nussbaum, 2021; Milusev, Rieben and Sorvillo, 2022).

2.6.2. Participation of the endothelial glycocalyx in heparin metabolism

Heparin chains, being either anticoagulant or not, are therapeutic exogenous GAGs that will as such be naturally exchanged with, or incorporated in, the endogenous glycans of the glycocalyx. Therefore, upon heparin injection, a part of heparin will bind to the glycocalyx; the size of this percentage is dependent on both the structure and the length of the heparin chains, as well as the structure of the glycocalyx. This has the following two implications: firstly, after heparin injection, heparin's glycan chains will be incorporated with the chains of the glycocalyx, it will however become at some point saturated and its incorporating capacity will be lowered. Gradually during the course of administration, the exchanges will be modified quantitatively, indicating that heparin's pharmacokinetics will no longer be the same since the time of the first administration, after numerous administrations (Bal Dit Sollier, Berge and Drouet, 2016). Secondly, after injection of LMWH, which is a combination of heparin chains, with and without anti – coagulant function, with different lengths and which are variably incorporated in the glycocalyx: longer chains, regardless of whether they possess the pentasaccharide sequence or not, are easier to incorporate within the endothelial glycocalyx in comparison with short chains. Therefore, from a pharmacokinetic point of view, heparin's antithrombin (AT) function will circulate for a shorter period of time than its anti – Xa activity (Laforest *et al.*, 1991).

A preparation with very short chains consisting solely of the pentasaccharide sequence, like fondaparinux, demonstrates minimal incorporation with the glycocalyx, indicating that the pharmacokinetics of reduction in the bloodstream will rely minimally on the glycocalyx, but rather on the renal excretion, the alternative form heparin elimination. Heparin's pharmacokinetics is further affected by the glycocalyx structure and composition. This justifies the alterations in heparin's pharmacokinetics in pathologies in which there is evidence of glycocalyx damage, such as cancer, especially under chemotherapy treatment (Carrier *et al.*, 2009), polytrauma (Malinoski *et al.*, 2010), sepsis (Dörffler-Melly *et al.*, 2002), pregnancy (Fox *et al.*, 2008), acute coronary syndromes (Montalescot *et al.*, 2003), as well as major burns and diabetes (Lin *et al.*, 2011). These alterations in pharmacokinetics are in line with the higher

incorporation of chains in the injured glycocalyx, ergo the measurement of reduced circulating anticoagulant activity (anti – Xa levels) than expected, given the dose injected and patient weight.

As such, there are related functions of heparin that are dissociated from its "classical" AT cofactor anticoagulant activity, but will be involved in its antithrombotic function, its anti – inflammatory function and in the restoration of the activities of the endothelium, i.e. flow regulation and permeability.

2.6.3. Glycocalyx – related and glycan – related activity of heparin

a. Anti – inflammatory effect

Through the glycocalyx, heparin is involved in leukocyte transendothelial passage and inflammatory cytokine transendothelial as well as endothelial effects (Rao *et al.*, 2010). This dissociation between the anti – inflammatory and anticoagulant function of heparin has been displayed especially with the use of forms of heparin with no anticoagulant activity, e.g. desulphated heparin, taking into account that the sulfation of numerous residues within the pentasaccharide sequence is in part responsible for the sequence's specificity, leading to its specific affinity for the AT molecule. This effect of heparin in the regulation of the interaction of leukocytes in the inflammatory response is attributed to the heparin glycan chains being incorporated in the endothelial glycocalyx. Upon its saturation, however, the anti – inflammatory property does not increase by either increased or repeated doses, in contrast with the circulating anticoagulant activity, that relies on plasma concentration and as such, the administered dose. This divergence of the dose reliance between the anti – inflammatory and the anticoagulant activity of heparin was one way to suggest that the anti – inflammatory activity is not associated with the classical anticoagulant impact of LMWH preparations (Downing *et al.*, 1998; Kevane *et al.*, 2017).

b. Anti – cancer effect

Regarding this property, it is appropriate to differentiate the antio – angiogenic from the anti – metastatic activity.

Several growth factors, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) in particular, display in their protein sequence heparin – binding sequences. Upon analysis of these growth factors' effect, either in presence or absence of heparin, heparin's very high affinity for these factors lowers their angiogenesis capability. Heparin's anti – angiogenic property does not include an impact on the glycocalyx and on these occasions, this property is dose – dependent with the administered heparin. Again, long heparin chains demonstrate greater suppressing effect on the activity of angiogenic factors and this is a glycan function (Mousa and Petersen, 2009).

A remarkable amount of work has been conducted to show that the anti – metastatic effect of heparin derives from its anticoagulant activity, especially through inhibition of thrombin. The work, however, that has evaluated modified heparin preparations, which are deleted of their AT cofactor activity as is evident in residue desulphation for example, even more so within the pentasaccharide sequence, indicates that these preparations are at the very least as effective as heparin preparations in animal models of the metastatic implantation of injected tumor cells (Stevenson, Choi and Varki, 2005; Kevane *et al.*, 2017). This function seems to be associated with the saccharide nature of heparin chains rather than their anticoagulant function, as is clearly evident from the comparisons between fondaparinux, UFH and LMWH. The investigated heparin in two studies was tinzaparin, a LMWH with a high percentage of chains containing more than 18 saccharides (Stevenson, Choi and Varki, 2005; Kevane *et al.*, 2017). This suggests that the anti – metastatic function of this LMWH does not greatly differ from that of UFH. Upon comparison with fondaparinux, however, which consists solely of five saccharides forming the AT cofactor sequence, the anti – Xa anticoagulant activity is very potent but an anti – metastatic effect is absent. A part of the anti – metastatic activity is attributed to the prevention of P – Selectin's interacting with its ligands, which like P – Selectin glycoprotein ligand – 1 (PSGL-1), are glycoprotein in nature (Stevenson, Choi and Varki, 2005; Kevane *et al.*, 2017).

c. Surface effect

Heparin's glycan nature and its highly negative charge give this molecule a high affinity for matrix surfaces. The heparin chain deposit on these surfaces induces their passive status towards elements of cellular response, e.g. leukocytes and platelets and molecular response, e.g. vWF, fibrinogen, etc, leading to a lower prothrombogenic potential. On occasions where blood comes in contact with wide artificial surfaces, heparin use is appropriate, as was the case for the anticoagulation of hemodialysis circuits in patients submitted to chronic antivitamin K treatment. This is still applied in hemodialysis patients requiring direct oral anticoagulants, as for example patients with concomitant atrial fibrillation.

Similarly, a study of the trials comparing heparin versus non – glycanic anticoagulants in coronary revascularization procedures for acute coronary syndromes applies: firstly, there have been studies comparing enoxaparin against fondaparinux, which is not a glycan chain, such as the OASIS-5 and OASIS-6 trials on acute coronary syndromes with or without ST-segment elevation. A modification of the protocol reinstated the administration of heparin at the time of the angioplasty procedures in the group receiving fondaparinux in order to prevent catheter – related thrombosis (Yusuf *et al.*, 2006a, 2006b). Likewise, in the trials evaluating bivalirudin in acute coronary syndromes, such as the HORIZONS – AMI trial, no material – related thrombosis (specifically acute stent thrombosis) was reported in the patients receiving bivalirudin also receiving additional heparin at the time of the procedure of angioplasty, while the respective rate of the patients receiving solely bivalirudin was significantly higher (Dangas *et al.*, 2011); this finding was reproduced in the EUROMAX trial (Steg *et al.*, 2013).

3. Heparin and thromboinflammation in the context of sepsis

3.1. Definitions

Sepsis is defined as a complication occurring when the human body releases host-defense mediators into the circulation in an attempt to counter infection. Systemic inflammatoriy responses activated as a result constitute this condition potentially lethal, responsible for 11 million sepsis – related deaths during the course of a single year and affecting a gross estimate of 48.9 million patients throughout the world (Rudd *et al.*, 2020). When organ dysfunction ensues as a corollary of a septic response, the term severe sepsis is applied. Sepsis survival rates still remain unsatisfyingly low (Kaukonen *et al.*, 2014) and even in chance of survival, patients frequently experience a deterioration in their quality of life (Williams, 2012).

Despite the immune system being currently not considered the sole mediator of sepsis pathogenesis, its role is still pivotal in that it initiates pathogen recognition and systemic inflammatory response. The complement and coagulation systems, however, have found to be intertwined (Amara *et al.*, 2008; Levi and van der Poll, 2010; Engelmann and Massberg, 2013), with coagulation derangement being noted in virtually all sepsis patients, varying from a subclinical prolonged clotting time to profound disseminated intravascular coagulation (DIC) (Levi *et al.*, 1993; Levi, 2008). Bibliography findings increasingly support (Engelmann and Massberg, 2013) that the interdependence between coagulation and inflammation – termed as thromboinflammatory response (Blair *et al.*, 2009) and coined by Ekdahl et al as thromboinflammation (Ekdahl *et al.*, 2016) – is much deeper and the two linked phenomena are major sentinels in sepsis pathophysiology. The observation that bleeding complications' continued occurrence despite the use of multiple anticoagulants with varying pharmacodynamics further cements this rationale (Zarychanski *et al.*, 2015; Fan *et al.*, 2016; Umemura *et al.*, 2016; Rhodes *et al.*, 2017), with heparin having been first trialed in a sepsis treatment regimen as soon as 1966 (Martinez, Fernandez and Vazquez-Leon, 1966).

In short, thromboinflammation takes place when the immune systems' cascade systems become activated and as a result propagate blood and EC activation; throughout the course of this process, there are numerous points of cross – talk (Markiewski *et al.*, 2007).

3.2. Pathophysiology of thromboinflammation in sepsis

3.2.1. Recognition molecules

This complex process enorchestrates several factors which are able to turn a small trigger of the cascade system, through amplification or interaction, to clinically evident thrombophlebitis or, in worse scenarios, to serious microvasculature injury of the organs and tissues affected.

Numerous recognition molecules are integrated in the intravascular cascade systems of the innate immune system, which are potent thromboinflammation triggers within the classical, the alternative and lectin pathway of complement (Nilsson, Teramura and Ekdahl, 2014; Ekdahl *et al.*, 2015). Firstly, the recognition molecule within the classical pathway (CP) is the C1q, binding to immunoglobulins, to pentraxins and molecules, e.g. lipopolysaccharide (LPS), DNA and heparin, which carry a negative charge. C – reactive protein (CRP) and pentraxin – 3 recognize phospholipid structures and pathogen – associated molecular patterns (PAMPS). Secondly, within the alternative pathway (AP), complement activation is a corollary of C3 spontaneous hydrolysis following either tissue injury or infection and is therefore highly non – specific. Finally, the recognition process through the lectin pathway (LP) is conducted by mannose – binding lectin (MBL), ficolins -1, -2 and -3 and collectins 10/11; non – self structures are recognized by all of them on the pathogens' surface and the LP of complement is subsequently triggered. Activation of these three pathways will ultimately lead to release of C3a and C5a anaphylatoxins, which recruit monocytes and polymorphonuclear cells (PMNs).

The contact system's primary recognition molecule is Factor XII (FXII). Heparin binds to the D5 domain of high – molecular – weight kininogen, which in turn binds to plasma kallikrein, inducing indirectly increased FXII activity. As such, heparin functions as a part of the innate

immune system, as it results in target sequestration by clots of fibrin and bound platelets inducing phagocytosis and ultimately, microorganism elimination (Frick, Björck and Herwald, 2007). Under conditions characterized by stress, e.g. trauma, hypoxia, ischemia or sepsis, the cells express cytokines, chemokines and TF, functioning indirectly as recognition molecules that emit altered – self signals. In a similar fashion such as the one described regarding the three complement pathways, activation of the two coagulation pathways, i.e. the TF and contact pathway, also leads to thrombin generation, triggering as such formation of fibrin and activation of platelets.

The grade and siting of a thromboinflammatory lesion are highly dependent on the vessels' EC lining. Under normal circumstances, both anti – thrombotic and anti – inflammatory substances are expressed in the endothelium, such as endothelial – derived developmental endothelial locus – 1 (Del – 1) which antagonizes leukocyte endothelium adhesion and the NTPDase CD39 which hinders accumulation of platelets (Croix *et al.*, 1996; Choi *et al.*, 2008).

3.2.2. Neutrophil Extracellular Traps, platelets and points of cross – talk between the coagulation and the immune system

Following vascular injury, proteins of the subendothelial matrix, such as von Willebrand factor and collagens, are substantially released; these interact with certain receptors on the platelet cells' surface and through a complex process, the description of which exceeds the purpose of the present thesis, recruitment of platelets on the damaged site occurs. Upon initial tethering, the following activation and adherence to the wall of the vessels results in intracellular granules releasing potent platelet agonists, such as ADP, propagating activation of paracrine platelets and ongoing binding, a sequence termed as platelet aggregation; through continuous platelet activation, adhesion and aggregation, a thrombus quickly grows in size (Moog *et al.*, 2001; Grüner *et al.*, 2003; Massberg *et al.*, 2003; Bergmeier, Chauhan and Wagner, 2008; Furie and Furie, 2008; Mackman, 2008). Platelets are also implicated in the cross – talk between the immune system and the coagulation pathway; within a forming clot, the recruited innate immune cells' functions are regulated by platelets and products of the coagulation pathway.

For example, mediators released by platelets, more specifically CXC – chemokine ligands (CXCL) CXCL1, CXCL4, CXCL5, CXCL7, CC-chemokine ligands (CCL) CCL3, CCL5, CCL7, CD154 of the CD40 ligand and a ligand for triggering receptor expressed on myeloid cells 1 (TREM1), strengthen the microbicidal activity of leukocytes (Haselmayer *et al.*, 2007; Yeaman, 2010; Semple, Italiano and Freedman, 2011; Weber and Noels, 2011). Likewise, innate immune cell expression of PARs is followed by their activation by factor Xa and thrombin, promoting dendritic cell proinflammatory outside-in signaling (Coughlin, 2005; Niessen *et al.*, 2008). The pathological activation of immune cells and intravascular thrombus formation, the "vicious cycle" of inflammation and blood coagulation, is a feature of systemic infections, including sepsis (Charles T Esmon, 2005).

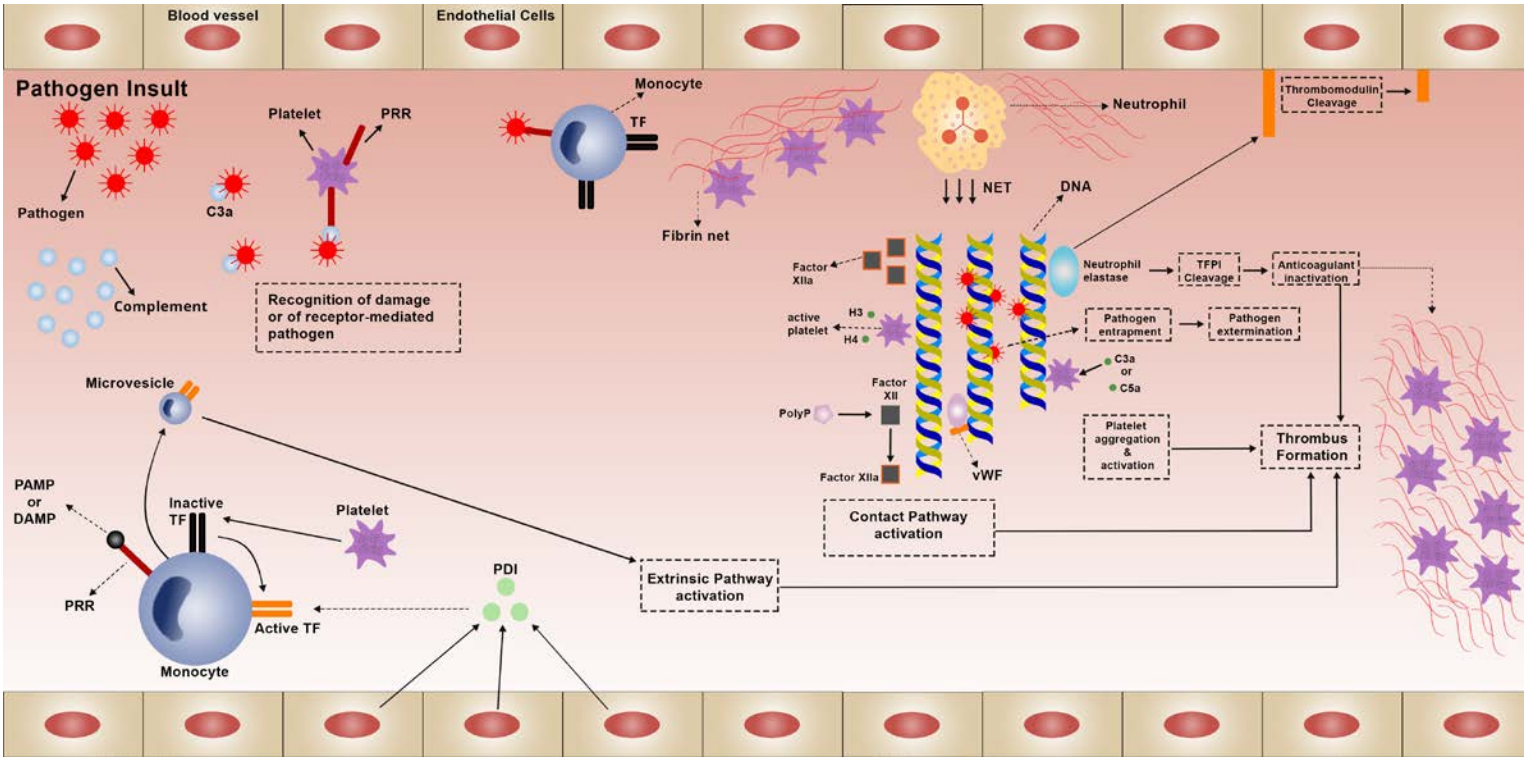
Production or otherwise activation of numerous host molecules associated with thrombosis is also conducted by neutrophils and monocytes. For instance, through the course of a thrombus development within a blood vessel, monocytes and monocyte – released microvesicles express TF, therefore promoting blood coagulation (Rivers, Hathaway and Weston, 1975; Giesen *et al.*, 1999; Müller *et al.*, 2003).

Another point of cross – talk between the two systems lies in the neutrophils' participation, traditionally considered as sentinels of the immune system; however, upon being activated, they produce a matrix of histones and DNA, constituting particles referred to as Neutrophil Extracellular Traps (NETs) and potentiating thrombosis (Brinkmann *et al.*, 2004). NETs entrap and increase neutrophil – mediated extermination of invading extracellular pathogens, while dealing but minimal damage to host cells. NETs possess a high antibacterial function, by employing neutrophil elastase, pentraxin, lactoferrin, peptidoglycan recognition protein 1 (PGLYRP1), matrix metalloproteinase 9 (MMP9) and myeloperoxidase (Dziarski *et al.*, 2003; Cho *et al.*, 2005; Jaillon *et al.*, 2007). Besides that, they further display prominent procoagulant activity: the proteolytic activity of neutrophil elastase stimulates coagulation (Massberg *et al.*, 2010); neutrophil elastase deposited by NETs also induces the inactivation and degradation of the anticoagulant molecule TFPI; platelets adhering to the neutrophils' surface augment formation of NETs and promote TFPI cleavage by neutrophil elastase; neutrophil serine

proteases stimulate the TF activated extrinsic pathway of coagulation. As such, neutrophil – platelet conjugates directly promote coagulation through augmentation of intravascular TF activity (Engelmann, Luther and Müller, 2003). NETs bound on platelet surface have also been found to induce their activation in the context of deep vein thrombosis (Fuchs *et al.*, 2010). Additionally, numerous other natural anticoagulants are also degraded by neutrophils. Neutrophil oxidases observed on NETs, for example, inactivate thrombomodulin, also cleaved by neutrophil elastase (Takano *et al.*, 1990; Glaser *et al.*, 1992). NETs also possess extracellular nucleosomes stimulating the contact pathway of coagulation, leading therefore to the formation of fibrin (von Brühl *et al.*, 2012). Finally, the aforementioned histones contained in NET extracellular nucleosomes are capable of platelet activation through Toll – like receptor 2 (TLR2) and TLR4, potentiating thrombosis (Xu *et al.*, 2009, 2011; Semeraro *et al.*, 2011).

Not only do platelets – which are per se basic clot components – augment coagulation by immune cell participation, they further stimulate thromboinflammation by several alternative mechanisms. One such example is tissue factor expression upregulation on innate immune cells, especially on monocytes, by platelets. Following a response to bacterial pathogens, platelets engage in a phenomenon referred to as NETosis, i.e. binding to neutrophils and initiation of NET formation (Clark *et al.*, 2007; Massberg *et al.*, 2010), a process as of yet not fully elucidated, although β-defensins excreted by activated platelets seem to play a substantial role (Kraemer *et al.*, 2011). NET formation does not rely exclusively upon platelet presence (Brinkmann *et al.*, 2004); platelet recruitment and activation can be initiated by NET histone components, particularly histones H3 and H4, via a feed – back mechanism (Fuchs *et al.*, 2010; Xu *et al.*, 2011). Finally, platelets release damage-associated molecular patterns (DAMPs), e.g. Protein disulfide isomerase (PDI), capable of initiating expression of tissue factor within a thrombus, a process that may promote thromboinflammation (Reinhardt *et al.*, 2008). The overall process of thromboinflammation is depicted in Figure 1.

Figure 1. Process of thromboinflammation.



In intact blood vessels, innate immune cells protect hosts from altered – self and non – self through stimulation of cell – specific prothrombotic pathways. For instance, stimulation of the extrinsic pathway of coagulation is attributed to the expression and activation of intravascular TF on sites of pathogen exposure by monocytes and monocyte microvesicles, as a corollary of response to PAMPs and/or DAMPs. Triggering of the contact pathway of coagulation is conducted by the activation of factor XII by platelet – derived PolyPs. The supportive activity of platelets in the thromboinflammatory process also includes production of fibrin by platelet-/endothelial cell – derived PDI (possibly as a result of TF activation). Activated platelets also augment thromboinflammation via activation of complement components C3a and C5a. Thromboinflammation is further stimulated by NETs, consisted by a matrix of DNA and histones, via several mechanisms, e.g. direct factor XII – dependent coagulation pathway activation, von Willebrand factor binding and recruitment of platelets, activation of platelets by NET histones H3 and H4, TF binding and resulting activation of the extrinsic pathway of coagulation and, finally, inactivation of endogenous anticoagulants TFPI and thrombomodulin through cleavage by neutrophil elastase or oxidization by myeloperoxidase (not depicted).

DAMP – damage-associated molecular pattern; NET – Neutrophil Extracellular Trap; PAMP – pathogen-associated molecular pattern; PDI – protein disulfide isomerase; PolyP – polyphosphate; PRR – pattern recognition receptor; TF – tissue factor; TFPI – tissue factor pathway inhibitor; vWF – von Willebrand factor

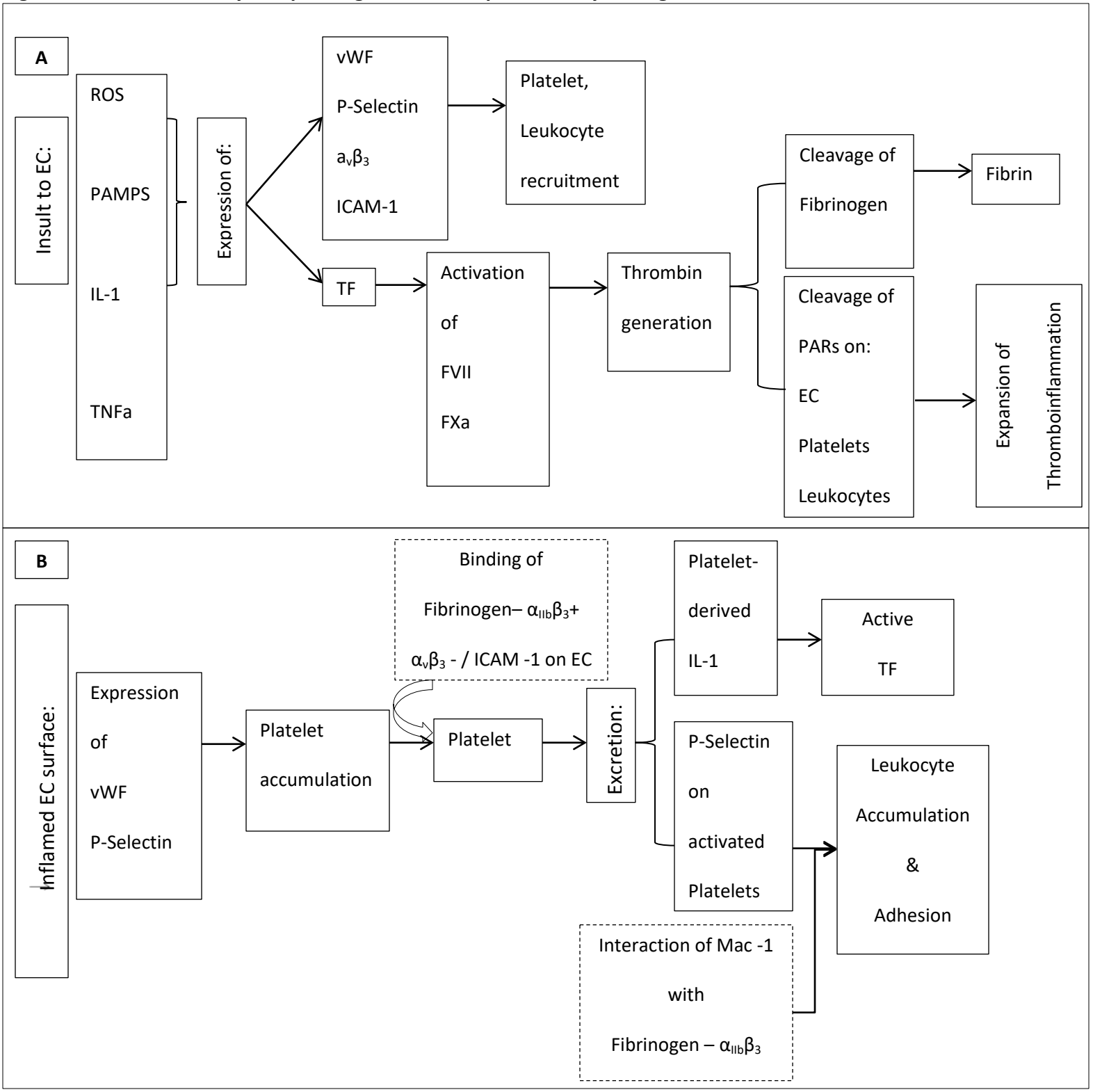
3.2.3. Regulation of thromboinflammation in the endothelium

The anti – inflammatory, anticoagulant and antiplatelet properties of the EC preserve vascular health (Aird, 2005). The anti – adhesive nature of the endothelium is attributed to three pathways, which are the nitric oxide (NO) pathway, the prostacyclin – or prostaglandin I2 (PGI2) – and the CD39/ectoadenosine diphosphatase (ecto-ADPase) pathway. The platelet agonist adenosine triphosphate/adenosine 59-diphosphate (ADP) is scavenged by CD39/Ecto-ADPase (Jin, Voetsch and Loscalzo, 2005). Apart from inhibiting platelet activation, PGI2 and NO further protect homeostasis through the following functions: NO downplays expression of P – selectin on the endothelial surface, chemokine expression, transcription of adhesion molecules such as E – selectin, VCAM-1, and ICAM-1, therefore reducing recruitment of leukocytes to the vessel wall. PGI2 decreases leukocyte adhesion, activation, extravasation and ergo inflammation. The endothelium also exerts certain functions to neutralize a – thrombin: binding of AT to GAGs on the EC surface and inactivation of a – thrombin (IIa), FXa and numerous coagulation proteases; switching of thrombin within the microcirculation from a procoagulant to an anticoagulant state through binding of the endothelial protein C receptor (EPCR) and endothelial integral membrane protein thrombomodulin (TM). Binding is followed by cleavage and activation of protein C (PC) by thrombin, producing activated protein C (APC), inactivating through proteolysis factors FVa and FVIIIa, acquiring anticoagulant property. In case of a remaining APC – EPCR bond, PAR1 triggering of signals occurs to promote cytoprotection and endothelial barrier activity. Additionally, increased quantities of TFPI are expressed by quiescent EC, inhibiting the conjugation of the TF/FVIIa complex leading to coagulation triggering and production of thrombin (C T Esmon, 2005; Jin, Voetsch and Loscalzo, 2005).

However, in sepsis, EC homeostasis is deranged by humoral mediators, as demonstrated in Figure 2 at the end of the paragraph. Bacterial cell wall components, i.e. pathogen – associated molecular patterns (PAMPs) induce endothelial surface pattern recognition receptor (PRRs) activation. This leads to production of cytokines, specifically tumor necrosis factor – 1 (TNFa) andIL-1, to elevated expression of adhesion molecules, e.g. P – Selectins, avb3, ICAM-1 and von Willebrand factor (vWF) and ultimately, to recruitment of platelets and leukocytes. Furthermore, activated EC express TF, promoting activation of FVII and FXa and therefore

thrombin generation. Fibrinogen cleavage by thrombin produces fibrin protease – activated receptors on the surface of plaletets and leukocytes, proliferating thromboinflammation. Regarding platelet and leukocyte recruitment on the inflamed EC lining, initiation is conducted by P – Selectin and vWF expression on the damaged EC surface, a condition in favor of platelet accumulation. Fibrinogen – integrin $a_{IIb}\beta_3$ complexes bind to ICAM-1 or $a_v\beta_3$ on EC, leading to continuous adhesion of platelets, releasing several agents that modify the EC adhesive and chemotactic status. Platelet – derived IL – 1 stimulates active TF expression, while expression of P – Selectin stimulates, mostly through macrophage-1 antigen (Mac-1) interaction with GPIb–GPV–GPIX and fibrinogen–$a_{IIb}\beta_3$ complexes, resulting in accumulation of leukocytes and their subsequent adhesion. Bacterial endotoxin also propagates TF expression and increases the levels of plasminogen activation inhibitor 1 (PAI-1), inhibiting therefore fibrinolysis and strengthening the procoagulant state of the EC lining. EC derangement is, in conclusion, associated with downregulation of major sentinels of the coagulation system under homeostasis conditions. Of note, immune complexes and toll-like receptors (TLR), such as TLR-4, activate platelets and further bridge the coagulation, contact and complement systems (Cognasse *et al.*, 2015). Their traditional role in the process of hemostasis is therefore transcended, as they also function as innate immune cells.

Figure 2. Proinflammatory and procoagulant effects produced by damaged endothelial cells.



Panel A displays the sequence of endothelium perturbation and **Panel B** the recruitment of platelets and leukocytes on damaged endothelium surfaces. EC: endothelial cells; ICAM 1 – intercellular adhesion molecule 1; IL-1: interleukin 1; Mac-1: macrophage-1 antigen; PAMPs: pathogen-associated molecular patterns; PARs: protease-activated receptors; ROS: reactive oxygen species; TF: tissue factor; TNFa: tumor necrosis factor-1; vWF: vonWillebrand factor.

3.2.4. Thromboinflammation promotion by tissue factor

In a study involving mice exposed to endotoxin, inhibition or low expression of TF achieved by administration of pharmacological agents was associated with a reduction of thromboinflammatory processes and mortality rates (Pawlinski *et al.*, 2004). TF production is mainly conducted by cells enveloping the wall of the vessels, specifically fibroblasts and pericytes (Østerud and Bjørklid, 2006). By binding to and activating factor VII (FVIIa), TF potently stimulates coagulation (Mackman, Tilley and Key, 2007) and is now considered a major factor in the initiation and preservation of the thromboinflammation, not only in sepsis but also in several alternative thromboinflammatory diseases (Erlich *et al.*, 2000). Monocytes are additionally a significant source of blood – borne TF, not only producing but also expressing TF (Osterud, 1998). Notably, numerous thromboinflammatory disorders display an association with circulating monocyte expression of TF, one of them being Gram – negative sepsis (Drake *et al.*, 1993; Lupu *et al.*, 2005; Osterud and Bjorklid, 2012). As soon as 1974, evidence has indicated a potential participation of platelets and neutrophils in TF production, but whether this amounts to significant levels remains as of yet controversial (Osterud and Bjorklid, 2012).

However, in a preclinical model of endotoxemia, deleting TF from EC reduced neither production of a – thrombin nor mortality, suggesting that the dominant TF sources in vivo responsible for propagating coagulation are non – endothelial (Pawlinski *et al.*, 2010). Solely preventing TF, particularly in humans, does not potently inhibit generation of a – thrombin and inflammation (Abraham *et al.*, 2003); therefore, the existence of additional pathways is a plausible assumption.

An alternative pathway stimulating a – thrombin generation is the contact system pathway of coagulation. Exposure of molecules bearing a negative charge, such as inorganic polyphosphates (PolyPs) released by exposed DNA/RNA from apoptotic or damaged cells or by platelets, activates the coagulation cascade (Gajsiewicz, Smith and Morrissey, 2017). Negatively charged surfaces envelop the bacterial cell wall and can induce contact factor activation (DeLa Cadena *et al.*, 1991), indicating that in sepsis this pathway may partake in the coagulation

cascade's activation (Tapper and Herwald, 2000). Moreover, generation of thrombin can be augmented by thrombin itself by means of positive feedback loops (Matafonov *et al.*, 2011).

3.2.5. Thromboinflammation coordination by a – thrombin

This molecule is essential in the pathogenesis of thromboinflammation and potentially for the development of future therapeutic methods due to the fact that, by cleaving numerous substrates, it induces pro- and anti- thrombotic, -coagulant and –inflammatory actions (Huntington, 2005). A significant number of those effects is attributed to PARs (Coughlin, 1999). Plasmin activates PAR1 and PAR4, while thrombin and cathepsin activate PAR3, in addition to the first two. Tryptase, trypsin, FVIIa and FXa activate PAR2, while FXa additionally may activate PAR1 (Camerer *et al.*, 2002; Borensztajn, Peppelenbosch and Spek, 2008; Adams *et al.*, 2011). Upon PAR cleavage on platelets by a – thrombin, several proinflammatory molecules, such as growth factors and chemokines, are released (Coppinger *et al.*, 2004), while granule contents are also released, more specifically a – thrombin itself, ADP, the CD40 ligand, P – selectin and serotonin (Lopez *et al.*, 2015) and thromboxane A2 is produced (Shankar *et al.*, 2006). Via PAR1 and PAR4, a – thrombin promotes platelet procoagulant function (Fager *et al.*, 2010). Platelet integrin $\alpha_{IIb}\beta_3$ activation by a – thrombin additionally propagates swift platelet accumulation and although a – thrombin stimulates both EC and platelets, the latter seem crucial in neutrophil recruitment unto localized endothelial lesion sites (Kaplan *et al.*, 2015).

Moving on now to the stimulation of EC by a – thrombin, a number of its proinflammatory properties is attributed to its activating EC via PAR1 proteolysis, resulting in TF expression, Weibel – Palade body mobilization and finally, in increased P – selectin expression and vWF release (Tull *et al.*, 2012). Additionally, a – thrombin induces adhesion molecules' increased expression, such as E – Selectin, VCAM – 1 and ICAM – 1 (Okada *et al.*, 2006) and release of cytokines, chemokines and growth factors (Coughlin, 1999). In summary, a – thrombin exerts numerous functions (Michelson *et al.*, 1991; Bajzar, Morser and Nesheim, 1996; Huber-Lang *et al.*, 2006; Bouton *et al.*, 2012; Krisinger *et al.*, 2012; Petrey and de la Motte, 2016) within the vascular system (Table 1) by cleaving key substrates within the coagulation, fibrinolytic and

complement cascades or by activating multiple cell types, e.g. EC, platelets, leukocytes, vascular smooth muscle cells and fibroblasts (Furuhashi *et al.*, 2008; Hsieh *et al.*, 2009; Kastl *et al.*, 2009).

Table 1. Actions of a-thrombin categorized in terms of final outcome.

COAGULATION		THROMBOSIS		INFLAMMATION	
Procoagulant	Anticoagulant	Prothrombotic	Antithrombotic	Proinflammatory	Anti-inflammatory
Activates FV (Fva)	Inhibits thrombin, FXa, FXIa activity through cleavage of Protease – nexin	Cleaves fibrinogen to fibrin	Inactivates ADAMTS13	Upregulates chemokine expression & ICAM-1, P-selectin through cleavage of endothelial PAR1	Activates protein C – APC
Activates FVIII (FVIIIa)	Inactivates FVa and FVIIIa through activation of protein C – APC	Activates FXIII (FXIIIa)	Cleaves GPV subunit of VWF receptor GPIb/V/IX	Increases expression of TNF, IL-1, IL-6, MCP-1 through cleavage of monocyte PAR1	Activates TAFI – TAFIa (and generates bradykinin & C5a)
Activates FXI (FXIa)		Activates platelets through cleavage of PAR1 and PAR4		Cleaves C5 (R947) & generates intermediate C5bT (& likely C5T)	Cleaves the inflammatory Inter-a-inhibitor heavy chain 1 (IaI-HC1) –associated hyaluronan (HA) matrix

ADAMTS13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 APC: Activated Protein C; ICAM-1: intercellular adhesion molecule 1; IL: Interleukin; MCP-1: Monocyte chemoattractant protein-1; PAR: protease-activated receptor; TAFI: Thrombin-Activatable Fibrinolysis Inhibitor; TNF: tumor necrosis factor;

3.3. Heparin administration in the context of sepsis

3.3.1. Heparin and NETosis

Heparin glycan chains, which bear a negative charge, will bind to positively charged NETosis filaments and as such exert protective actions against the cytotoxicity induced by said filaments (Wildhagen *et al.*, 2014). Simultaneously, they exhibit antithrombotic function that is not associated with the antithrombin cofactor anticoagulant effect.

Heparin ergo demonstrates antithrombotic action through limitation of platelet activation and degranulation, by inhibiting the binding of the von Willebrand Factor/ Factor VIII complex (Grässle *et al.*, 2014) and by hindering microparticle binding, especially those that carry TF (Grasso *et al.*, 2018), to the NETosis filaments (Fuchs *et al.*, 2010; von Brühl *et al.*, 2012).

The aforementioned effects will therefore elucidate the impact of heparin in pathologies, besides sepsis of course, implicating NETosis that, up till now, the scientific community attempted to attribute to their conventional anticoagulant activity. The administration of heparin in low doses in sepsis particularly (Squizzato *et al.*, 2016; Yamakawa *et al.*, 2016) is linked with a negating impact on NETosis per se, thus having a beneficial impact, which however is lost upon dose increase, inducing bleeding complications in association with heparin's classical anticoagulant activity.

Non – anticoagulant functions have further been reported in placental vascular pathologies (McLaughlin *et al.*, 2017). Experimental models indicate that NETosis is implicated in venous or arterial thrombosis in DIC. The neutralization of NETs in patients with cancer may hopefully lower morbidity and mortality as a result of cancer – induced thrombosis. In addition to its conventional anticoagulant actions, therefore, heparins, including LMWH display protective activity against the cytotoxity caused by NETosis (Stevenson, Choi and Varki, 2005; Mousa and Petersen, 2009; Megens *et al.*, 2012; Borissoff *et al.*, 2013; Döring *et al.*, 2015; Giaglis, Hahn and Hasler, 2016; Kevane *et al.*, 2017).

3.3.2. Anti – thromboinflammatory functions exerted by heparin

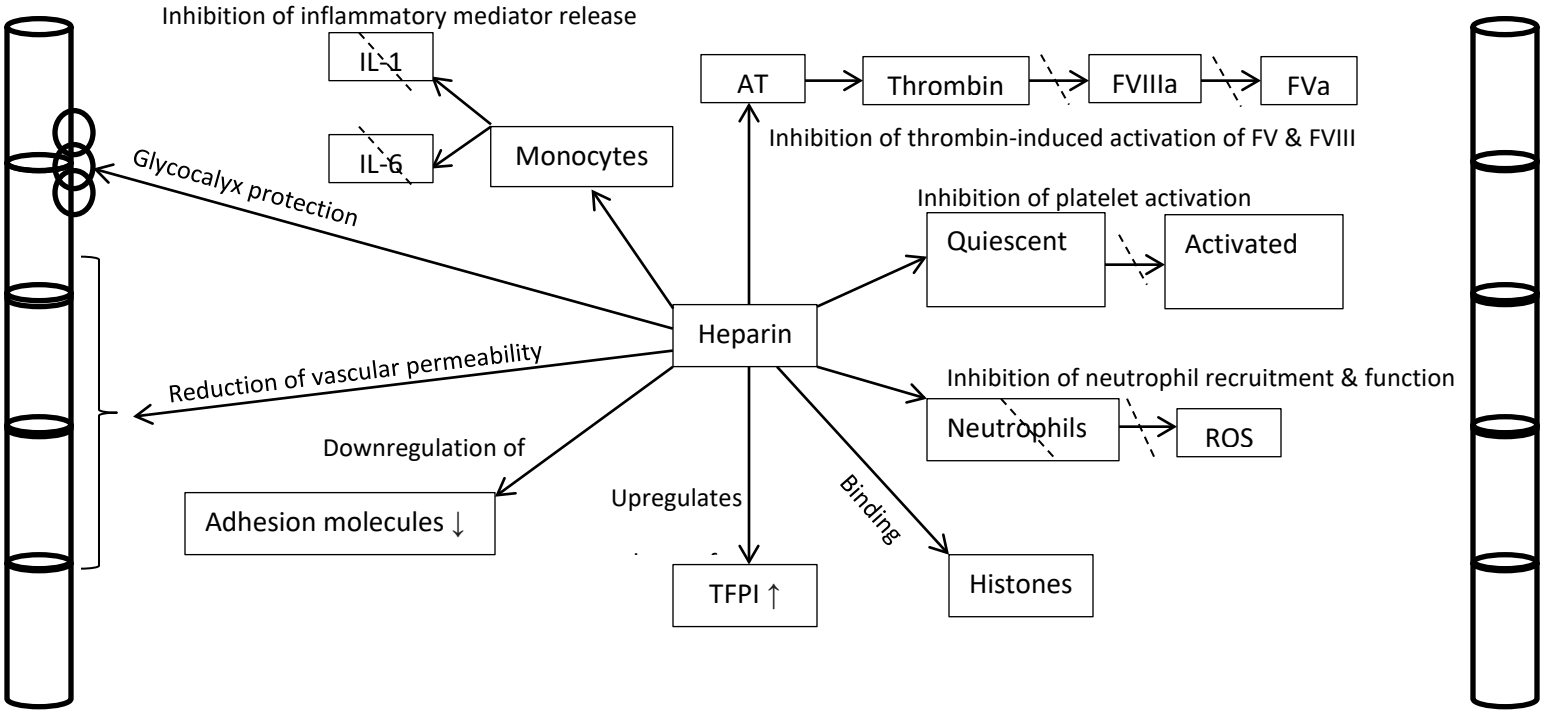
Following heparin's discovery in 1916, this GAG is consistently being administered in routine clinical practice, indicated first and foremost for the prevention of venous thromboembolism. Various mechanisms contribute to the anticoagulant property of heparin, but it is the inhibition, potentially reaching 1000 – fold levels, of thrombin and FXa via interplay with AT that is the most unique (Alban, 2005). Even though heparin's anticoagulant function was the first to be recognized and still the most clinically applicable, it has been found to exhibit such a wide range of properties that today, categorizing it solely as an anticoagulant would definitely be an understatement (Torri and Cassinelli, 2018). Specifically in the context of sepsis, heparin displays both protective anticoagulant and immunomodulatory activity; specifically its anticoagulant actions include, besides binding to AT, binding to heparin cofactor II, promoting TFPI release and inhibiting the thrombin – induced activation of FV and FVIII (Hirsh *et al.*, 2001; Hollenstein *et al.*, 2002; Alban, 2005). As far as its immunomodulatory function range is concerned, heparin was found to be associated with: pulmonary oedema prevention (Hiebert and Liu, 1990; Hocking, Ferro and Johnson, 1992; Meyer *et al.*, 1993); Angiogenesis inhibition (Brassart *et al.*, 1991); inhibition of neutrophil activity (Bazzoni *et al.*, 1993); reduction of eosinophil migration (Teixeira and Hellewell, 1993); decrease of platelet activation (Anaissie *et al.*, 1998); pulmonary hypertension downregulation (Darien *et al.*, 1998); inhibition of inflammatory LPS – induced mediators (Rex *et al.*, 2000; Blot *et al.*, 2002; Li *et al.*, 2012, 2014, 2015); neutrophil recruitment reduction (Eggimann, Garbino and Pittet, 2003; Ding *et al.*, 2011); binding to histones (Xu *et al.*, 2009; Fuchs, Bhandari and Wagner, 2011; Iba *et al.*, 2015; Alhamdi *et al.*, 2016); reduction of inflammation and mortality (Ding *et al.*, 2011) and reduction of lung inflammation by means of nuclear factor – kB (NF-κB) inactivation (Li *et al.*, 2013). Of note, apart from thromboinflammation – protective activity (Table 2), heparin evidently protects the EC glycocalyx from shedding via suppressing inflammation (Yini *et al.*, 2015) and reconstructing cell surfaces through syndecan – 1 mobilization (Nelson *et al.*, 2008), therefore ultimately maintaining vascular health (Figure 3).

Table 2. The anti – thromboinflammatory range of heparin functions.

ANTICOAGULATION	Binding to AT Binding to heparin cofactor II TFPI release stimulation Inhibition of thrombin – induced FV & FVIII activation
IMMUNOMODULATION	Inhibition of angiogenesis Pulmonary oedema prevention Neutrophil activity inhibition Eosinophil migration reduction Platelet activation decrease Pulmonary hypertension downregulation Neutrophil recruitment inhibition Histone binding Inflammation (and mortality) reduction Reduction of LPS – induced inflammatory mediators NF-κB inactivation with subsequent lung inflammation reduction

AT: antithrombin; LPS: lipopolysaccharide; NF-κB: Nuclear Factor-κB; TFPI: tissue factor pathway inhibitor.

Figure 3. Functions of heparin within the damaged endothelium in the context of sepsis.



AT: antithrombin; EC: endothelial cells; IL: interleukin; TF: tissue factor; TFPI: tissue factor pathway inhibitor; ROS: reactive oxygen species

3.3.3. Clinical trials integrating heparin in the treatment of sepsis

So far, either unfractionated heparin (UFH) or light molecular weight heparin (LMWH) have been put under scrutiny as potential therapeutic agents in patients under severe sepsis in three phase 3 RCTs (Bernard *et al.*, 2001; Warren *et al.*, 2001; Abraham *et al.*, 2003). A comparison between AT against placebo was conducted in the Kybersept study (n=2314) (Warren *et al.*, 2001); in the placebo arms, administration of low dose heparin prophylaxis demonstrated a trend towards decreased mortality, albeit not stastistically significant. The first study which actually displayed a benefit in survival amongst critically ill, sepsis patients was the PROWESS study (n=1690) by Bernard et al (Bernard *et al.*, 2001); APC was trialed as a host inflammatory response regulator and the finding is based on a subgroup analysis and therefore warrants testing. The OPTIMIST study (n=1754) (Abraham *et al.*, 2003) integrated recombinant TFPI in comparison with placebo in terms of potential benefit; mortality rates were found to be reduced following administration of UFH which was prescribed regardless of sepsis – related etiology. Of note, administration of heparin followed the randomization of the enrolled patients, possibly being more common among patients suffering from milder severity illness, also being prescribed taking into account patients' clinical need, therefore creating a potential source of selection bias. Homogeneity of patient groups was also an unknown parameter that warrants caution upon interpretation of the above findings. Based on the aforementioned studies alone, but in fact on any study investigating other compounds' efficacy, the possible therapeutic role of heparin in sepsis seems difficult to be determined due to either allocation or selection bias, or both. It is also difficult, on the other hand, to entirely exclude the potential benefit of heparin in sepsis.

In a retrospective propensity matched study (n=695) (Zarychanski *et al.*, 2008) a reduced 28 – day mortality was noted following intravenous administration of heparin within 48 hours after patient admission in the intensive care unit (ICU) (40.1% vs 44.2%, p=0.05) leading to the conclusion that early systemic heparin might be an effective treatment for patients under severe sepsis. These findings were corroborated in a post hoc analysis of human studies investigating anticoagulant medication as part of sepsis treatment (Polderman and Girbes, 2004). A prospective randomized double blind study, the HETRASE study (n=319) (Jaimes *et al.*,

2009), in which patients were allocated to either receive intravenous heparin by a continuous 500 IU/hr infusion with no bolus dose or placebo, concluded that heparin was safe with no increased bleeding risk, but failed to demonstrate a significant reduction in hospital length of stay, which was the primary outcome, but also in terms of mortality and organ failure scoring. The limitations of this study include: firstly, a heparin dose that may be inadequate in sepsis, since the treatment and control groups displayed no significant difference in clotting time assays. Secondly, mortality was not a primary outcome. Thirdly, a more heterogeneous population and patients suffering from less severe illness were enrolled in the study and finally, the same applies for patients with or without overt DIC. A recent post hoc subgroup analysis of a multicentre nationwide retrospective cohort study with 42 included ICUs (n=2663) (Yamakawa *et al.*, 2016) compared the therapeutic effect of any anticoagulant, i.e. heparin/heparinoid, rhTM, serine protease inhibitors or AT, in terms of mortality by categorizing patients according to DIC and Sequential Organ Failure Assessment (SOFA) scoring (Jones, Trzeciak and Kline, 2009). Upon discrepancy correction, anticoagulant treatment exhibited an association with reduced mortality only in subgroups with sepsis – induced coagulopathy and/or very severe illness. It is possible, therefore, to partially attribute the negative results of the HETRASE study to these findings.

Four reviews and meta – analyses have additionally investigated the potential role of heparin in sepsis treatment (Liu, Zhu and Ma, 2014; Wang *et al.*, 2014; Zarychanski *et al.*, 2015; Fan *et al.*, 2016). A shared finding of all four is the association of treatment with low doses of heparin with reduced 28 – day mortality in sepsis. In the study by Zarychanski et al (Zarychanski *et al.*, 2015), only trials in which patients were randomly allocated either to receive heparin or not were included; despite a 12% lower mortality risk with intravenous administration of heparin, the differing definitions of sepsis utilized through the long time period between the included trials (1983-2014) is a concern overshadowing the overall positive findings. In another study (Wang *et al.*, 2014), UFH was administered in the 40% of the study's patients, whereas either UFH or LMWH was administered in the rest. Prophylactic administration was associated with a 40% mortality reduction. Of note, the results regarding bleeding complications and mortality were on the most part derived from non – RCTs, possibly influencing the results due to the fact that

prescription of heparin was not randomized. Another meta – analysis (Fan *et al.*, 2016) has very recently presented that LMWH is an efficient and safe drug to be utilized in sepsis patients. The same conclusion was reached regarding also unfractionated heparin, in a meta – analysis published in January 2022 (Fu *et al.*, 2022) including 15 randomized controlled trials with a total of 2,617 patients evaluated the efficiency of UFH in adult patients with sepsis and found that it was associated with a reduced 28-day mortality rate [relative risk (RR)=0.82, 95% confidence interval (CI)=0.72-0.94, $p<0.05$].

In light of the above, so far the evidence regarding the place of heparin as a therapeutic modality in sepsis remains contradictory, it does however seem to exert beneficial effects. Through the course of its almost a century – long routine clinical practice, it has been utilized safely, with the most feared complication being a major haemorrhage. An additional adverse event to be taken into consideration is heparin – induced thrombocytopenia, even more so following the administration of UFH, carrying a ten – fold greater risk as opposed to LMWH (Linkins *et al.*, 2012). On the other hand, UFH displays more systematic action and predictable bioavailability, constituting a preferable choice amongst critically ill patients (Dörffler-Melly *et al.*, 2002; Priglinger *et al.*, 2003). Indeed, a percentage as high as 85% of sepsis patients displayed hypoperfusion and the subsequent poor absorption via the subcutaneous route and ultimately diminished bioavailability might have limited heparin's efficacy.

Of note, following the recent outbreak of the COVID pandemic, a disease that has definitely had a major impact not only in general healthcare but also in intensive care particularly, it has also been shown that this disease is closely intertwined with thromboinflammatory pathology. Numerous major factors in downregulated immunothrombosis, activated platelets, platelet-derived extracellular vesicles (pEVs) expressing HMGB1, PF4, soluble PF4, histones and NETs were found to bind to heparin due to their molecule's positively charged surface (Ebeyer-Masotta *et al.*, 2022). Adsorbents functionalized with endpoint – attached heparin were found to reduce HMGB1, histones, nucleosomes, pEVs, PF4 and activated platelets; this led to the authors proposing usage of heparin-functionalized adsorbents so as to prevent thrombotic complications in the clinical context of sepsis or COVID disease through the mechanism of

thromboinflammation central effectors' obliteration. A retrospective multicenter study (Ebeyer-Masotta *et al.*, 2022) investigated the effect of anticoagulant treatment on immunothrombosis biomarkers in coronavirus disease and interestingly, observed no significant reduction of immunothrombosis in patients receiving LMWH, contrary to an association with improved survival rates, markers of cell death and reduced viral persistence, encouraging therefore administration of LMWH in COVID patients without contraindications. This observation happens to be in line with previous findings (Vasileiadis *et al.*, 2018; Papadakis *et al.*, 2022) that markers associated with endothelial activation or injury paradoxically did not improve despite patients' clinical improvement, suggesting that endothelial damage may actually still ensue long past a septic stimulus (Leclerc *et al.*, 2000). As predicted however, liberal use of LMWH has been associated with bleeding complication occurrence. Administration of LMWH was found to increase bleeding events (Al-Samkari *et al.*, 2020); additionally, in the INSPIRATION trial (Sadeghipour *et al.*, 2021), in which intermediate doses of enoxaparin, i.e. a LMWH, were compared against standard prophylactic doses, not only was no significant benefit in critically ill COVID patients observed, but they also found a tendency for increased major bleeding event occurrence in the intermediate – dose arm of the study.

Needless to say, the attending physician should take into consideration the most appropriate compound, timing, route and dose to administer heparin to make most of it within clinical practice and so far, these questions have not been concretely answered.

4. ***Heparin and hepcidin regulation in critical illness***

4.1. Hepcidin

This antimicrobial peptide with activity in innate immunity was described in 2000 – 2001 and its name derives from its hepatic origin, hence the "hep" and its antimicrobial function, hence the "cidin". It is also termed Liver – Expressed Antimicrobial Peptide 1 (LEAP – 1) (Krause *et al.*, 2000; Park *et al.*, 2001). Hepcidin expression was linked with iron metabolism when evidence showed that iron excess and the inflammatory agent LPS stimulated hepcidin expression contrary to iron depletion which led to its reduction (Pigeon *et al.*, 2001). It was not too long later that hepcidin's pivotal role in iron homeostasis was cemented when the inactivation of the hepcidin gene was associated with iron excess in the pancreas and the liver (Nicolas *et al.*, 2001). Additionally, excessive liver – specific expression of hepcidin in transgenic mice induced severe iron – deficiency anaemia even at neonatal levels (Nicolas, Bennoun, *et al.*, 2002), while its expression is further regulated by hypoxia, anaemia and inflammation (Nicolas, Chauvet, *et al.*, 2002; Nicolas, Viatte, *et al.*, 2002). Patients carrying homozygous mutations in the hepcidin gene (HAMP) suffer from severe juvenile hemochromatosis (Roetto *et al.*, 2003); in a mouse model of hemochromatosis, additionally, iron excess is inhibited by constitutive hepcidin expression (Nicolas *et al.*, 2003)

4.1.1. Structure of hepcidin

The human hepcidin gene is sited on chromosome 19 and encodes an 84 – aminoacid prepropeptide that is subjected to two sequential cleavages, firstly to cleave the signal peptide and secondly to generate the mature 25 – aminoacid peptide, i.e. Hepc – 25. One of the major prohormone convertases, furin, is the enzyme responsible for hepcidin's processing via recognition of the consensus sequence (QRRRRR↓DTHF) (Shike *et al.*, 2004; Valore and Ganz, 2008). The processing might involve more mechanisms, as there have been described three additional hepcidin N – terminal truncated forms of 20, 22, and 24 aminoacids, i.e. Hepc – 20, Hepc – 22 and Hepc – 24, their role and production however are as of yet not fully elucidated

(Park *et al.*, 2001; Anderson *et al.*, 2012; Chaithanya *et al.*, 2013). Mice possess two different genes, i.e. Hepc – 1 and Hepc – 2, out of which only the former partakes in the metabolism of iron (Nemeth and Ganz, 2006). Within the mature Hepc – 25, the NMR structure contains two short β – strands that are united by four inter-strand disulfide bonds with hydrophobic surface and cation charge (Jordan *et al.*, 2009).

4.1.2. The axis of hepcidin - ferroportin

The mature Hepc-25 peptide's function is control of dietary iron absorption in the duodenum and also its release from organs storing iron, mostly liver and spleen. This activity is attributed to its binding to the only cellular iron exporter, ferroportin (FPN) essential for iron transport from the cytoplasm to the circulating transferrin (Nemeth *et al.*, 2004; Hentze *et al.*, 2010). FPN expression is mostly conducted by enterocytes of the duodenum and macrophages of the spleen and liver that process iron – rich effete red blood cells (RBCs) (Liu *et al.*, 2005) and is regulated at a transcriptional (Drakesmith, Nemeth and Ganz, 2015) and a post – translational level (Muckenthaler, 2008; Sangokoya, Doss and Chi, 2013). It is regulated, however, on the most part by hepcidin; after binding to FPN, it induces its internalization and degradation in order to decrease systemic iron availability (Nemeth *et al.*, 2004, 2006; Fernandes *et al.*, 2009; Ganz and Nemeth, 2012; Qiao *et al.*, 2012). This process suggests that a higher serum hepcidin concentration results in lower exposed ferroportin levels and ergo lower iron availability. The regulation of hepcidin is therefore obviously central in clarifying and controlling iron homeostasis.

4.1.3. Cellular signals regulating hepcidin

Hepatocytes are the major source of hepcidin; different stimuli regulate hepcidin expression, but mainly iron status and inflammation.

The cellular pathway of hepcidin expression was identified from the groundbreaking findings of a study of a mouse model with liver – specific disruption of the SMAD4 gene which found severely reduced hepcidin expression levels and extremely high multi – organ iron accumulation (Wang *et al.*, 2005). SMAD4 partakes in the Bone Morphogenetic Protein (BMP) and TGF-Beta pathways; the function of BMPs to stimulate hepcidin in hepatocytes has been confirmed several times and it was found that BMP6 specifically correlates with body iron reserves in mice (Kautz *et al.*, 2008; Andriopoulos *et al.*, 2009; Meynard *et al.*, 2009). Liver BMP6, mostly produced by non – parenchymal hepatic cells, is now considered the major BMP to regulate iron metabolism in mice (Rausa *et al.*, 2015).

Two types of dimeric BMP – receptors are involved in the canonical BMP pathway: type I and type II. With the ligand present, the receptors form a complex and the type II, specifically BMPR2 and Actra2A, phosphorylate the type I receptors, specifically Alk2 and Alk3. This process stimulates the phosphorylation of SMAD1/5/8 which recruit SMAD4, translocating within the nucleus in order to bind the BMPR element's specific promoter (Miyazawa *et al.*, 2002). SMAD6 and SMAD7, which are known inhibitors of the SMAD pathway, are also involved in regulation of hepcidin (Vujić Spasić *et al.*, 2013). In a mouse model of Type I receptor hepatic deletion, especially Alk3, excessive levels of iron were observed (Steinbicker *et al.*, 2011). Both Alk2 and Alk3 were required for the maximal response of hepcidin to iron, whereas in Alk2 or Alk3 deficient mice, treatment with exogenous iron failed to augment expression of hepcidin. Alk3 seems to be stabilized by HFE (the "High Iron" gene) through inhibition of its ubiquitation and proteasomal degradation, promoting as such Alk3 expression on the cell surface (Wu *et al.*, 2014). The expression of hepcidin involves both Type II receptors; only in the context of both Type II receptors' deficiency is the iron dependent increase of hepcidin suppressed with subsequent severe iron excess (Mayeur *et al.*, 2014). A pivotal sentinel of this pathway is Hemojuvelin (HJV), a liver – specific coreceptor (Babitt *et al.*, 2006; Corradini, Babitt and Lin, 2009). The importance of the role of HJV within the pathway was evident by the observation that HJV mutations induce a strong hepcidin suppression and iron overload, suggesting the existence of a regulatory mechanism for this particular protein, probably due to Matriptase-2 (MT2), a liver – specific serine protease, also termed TMPRSS6 (Finberg *et al.*, 2008; Silvestri *et*

al., 2008; Zhang *et al.*, 2011; Zhao *et al.*, 2015; Frýdlová *et al.*, 2016), whose ectodomain interacts directly with HJV and cleaves its molecule, hindering BMP/SMAD signaling and reducing expression of hepcidin (Silvestri, Pagani and Camaschella, 2008). MT2 inactivation leads to hepcidin excess both in mice and humans, resulting ultimately in iron refractory iron deficiency anemia (IRIDA) (Du *et al.*, 2008; Finberg *et al.*, 2008; Folgueras *et al.*, 2008). Recent, but controversial, evidence indicates that MT2 further cleaves HFE, BMPR2, ActRIIA, Alk2, Alk3 and at a lower grade TfR2 and HJV (Wahedi *et al.*, 2017). This pathway seems to include participation of several additional proteins, e.g. FKBP12 (Colucci *et al.*, 2017), Endofin (Goh *et al.*, 2015), Neogenin (Enns, Ahmed and Zhang, 2012; Zhao *et al.*, 2016) and BMP binding endothelial regulator (BMPER) (Patel *et al.*, 2012).

Inflammation additionally controls hepcidin expression through the inflammatory cytokine IL-6 which binds a particular receptor and induces Janus Kinase 1/2 (JAK1/2) activation and in turn Signal Transducer And Activator Of Transcription 3 (STAT3) phosphorylation, followed by STAT3 translocation into the nucleus and binding to the STAT3 response element (STAT3-RE) in the hepcidin promoter, promoting transcription (Verga Falzacappa *et al.*, 2007). This inflammatory signaling sequence depends on the BMP/SMAD pathway to stimulate expression of hepcidin; indeed, stimulation of IL-6 does not produce a response in SMAD4-knockout mice (Wang *et al.*, 2005), whereas Activin B, which is stimulated by inflammation, seemingly connects the SMAD and STAT3 pathways (Besson-Fournier *et al.*, 2012, 2017; Canali *et al.*, 2016). Therefore, inflammation and iron excess function to increase hepcidin and lower iron availability, contrary to conditions mandating the suppression of hepcidin's expression due to higher iron supply requirements. Such conditions are hypoxia, involving hypoxia-inducible factor (HIF), anemia with increased erythropoiesis, involving Erythroferrone, Growth/differentiation factor-15 (GDF-15) and twisted gastrulation (TWSG) and pregnancy, which involves the sex hormones, e.g. testosterone, progesterone and 17beta estradiol (Sangkhae and Nemeth, 2017). Recent evidence indicates that in the context of Hepatocellular Carcinoma, the methylation of the hepcidin promoter may also be important for its regulation (Udali *et al.*, 2018). Even though hepcidin regulation has been increasingly understood, there are still some clarifications to be made in order to develop novel therapeutic modalities in conditions characterized by hepcidin

dysregulation, common amongst numerous human diseases, some of which very well affect the critically ill.

4.1.4. Dysregulation of hepcidin

As previously stated, the corollary of a misregulated hepcidin expression is body iron availability imbalance and it was categorized into two types of genetic diseases: primary and secondary hepcidin – related disorders (Ganz and Nemeth, 2011). The former type are attributed to differentiations in genes participating directly in the metabolism of iron, characterized by hepcidin dysregulation as their primary pathogenetic mechanism; these include genetic hypotransferrinemia (Trombini *et al.*, 2007; Bartnikas, Andrews and Fleming, 2011), genetic hemochromatosis (Pietrangelo, 2015; Powell, Seckington and Deugnier, 2016; Sivakumar and Powell, 2016), characterized by low hepcidin levels and on the other hand, iron refractory iron deficiency anemia (IRIDA), characterized by hepcidin excess (Du *et al.*, 2008; Finberg *et al.*, 2008; Melis *et al.*, 2008; Benyamin *et al.*, 2009). In the secondary hepcidin – related disorders, the genes that result in hepcidin expression and iron availability perturbation are outside of the iron homeostasis system. Those include β – thalassemia (Origa *et al.*, 2007; Tanno *et al.*, 2007), chronic kidney (Ashby *et al.*, 2009; Zaritsky *et al.*, 2009) and liver diseases (Fargion, Valenti and Fracanzani, 2011) and anemia of inflammation, with increased incidence among hematologic malignancies, a number of solid tumors, chronic infections and inflammatory disorders (Corwin and Krantz, 2000; de Mast *et al.*, 2010; Hashizume *et al.*, 2010; Hohaus *et al.*, 2010).

Several pharmacological strategies have been described in order to restore appropriate hepcidin levels, involving identification of hepcidin agonists and antagonists (Sebastiani, Wilkinson and Pantopoulos, 2016). The approaches to lower expression of hepcidin concern the inhibition of hepcidin activity in binding to FPN or interference of the BMP6/SMAD or the IL-6/STAT3 pathway and numerous of the agents developed are currently under clinical trialing (Liu *et al.*, 2016; Sebastiani, Wilkinson and Pantopoulos, 2016; Reichert *et al.*, 2017; Vyoral and Petrak, 2017). One of the compounds able to suppress hepcidin expression, heparin, may be promising thanks to its low cost, its proven safety and also thanks to the fact that it is a drug

widely known and routinely used in clinical practice and its efficacy is improved even upon deletion of its anticoagulant properties. Investigation of the mechanism with which heparin interferes with expression of hepcidin might contribute to clarifying iron homeostasis regulation.

4.2. The heparan sulfates

HS, also described in paragraph 2.1, is a linear sulfated GAG and is expressed by all animal cells, displaying a very low range of structural variations through its circa 500 million years of evolution. Its absence is not compatible with life (Lin *et al.*, 2000; Stickens *et al.*, 2005), a fact that mirrors the ability of HS to regulate the function of various proteins, participating in cell attachment, differentiation, invasion, migration as well as in morphogenesis, organogenesis lipid metabolism, blood coagulation and inflammation, therefore modulating many aspects in cell biology (Bishop, Schuksz and Esko, 2007).

HS chains generally form covalent bonds with core protein, forming the Heparan Sulfate ProteoGlycan (HSPG) (Sarrazin, Lamanna and Esko, 2011). The anomalies in HS biosynthesis or HSPG functionality are recognized causes of various diseases in humans (Li and Kusche-Gullberg, 2016). Between all proteoglycans that have been adequately investigated, only 17 evidently consist of HS, grouped in four main types, characterized by variable core protein sturctures.

Two of said groups contain HSPG linked with the plasma membrane, particularly the glycosylphosphatidylinositol (GPI) – anchored Glypicans and the transmembrane Syndecans. The third category includes variable excreted HSPG (involving agrin, perlecan and collagen XVIII). The fourth category includes the intracellular HSPG, serglycin in particular, which carries heparin chains that are able to contribute to the interplay with various factors and proteins, therefore the biosynthesis and the modulation of HS formation, elongation and modification are intimately related to HSPG biological activity. The biosynthesis of HS takes place within the

Golgi apparatus and implicates a series of reactions that produce a glucuronosyl-galactosyl-galactosyl-xylosyl (GlcAβ1, 3Galβ1, 3Galβ1, 4Xyl) tetrasaccharide that forms a covalent bond with a core – protein – serine residue. This tetrasaccharide – protein bond is the same for carrying heparin (HS), chondroitin (CS) or dermatan (DS) chains. Which type of GAG chain will be formed is determined during the following glycolysation step, which chooses the mechanism towards the production of the type chains. The biosynthesis of the HS is a very complex procedure that exceeds the purpose of the present thesis and includes the initiation, the elongation and the maturation by the addition of sulfates and the epimerization of the molecule, processes in which a total of 20 and more enzymes are involved.

So far, it is not known what dictates the size and structure of HS in different cells at different times during development but, if it is not performed physiologically, it results in pathologic phenotypes (Nadanaka and Kitagawa, 2008; Soares da Costa, Reis and Pashkuleva, 2017). Between the several proteins that interact with HS but heparin as well, are FGF and the BMPs (Wozney *et al.*, 1988) and a number of those were first recognized following affinity chromatography with heparin – sepharose beads (von Einem, Schwarz and Rudolph, 2010). Now, the involvement of HS and HSPG in the regulation of the BMP2 osteogenic activity is known; HS and HSPG sequester BMP2 at the cell surface and facilitate its interaction (Jiao *et al.*, 2007). Additionally, HSPGs have been shown to imitate the BMP co – receptor in C2C12 and PC12 cells (Kuo, Digman and Lander, 2010). Their cell surfaces play a triggering role in the signaling pathway of BMP2 and BMP4; they contribute to the formation of the signaling complexes via type II receptor subunit recruitment to BMP-type I receptor complexes (Kuo, Digman and Lander, 2010). The heparin binding site of BMP2 and BMP4 is located at the basic – residue – rich N-terminus of the mature protein (Ruppert, Hoffmann and Sebald, 1996; Choi *et al.*, 2010; Gandhi and Mancera, 2012), but this sequence is not present in BMP6, which displays greater length and reduced basic residue density. In preliminary studies applying recombinant BMP6 and synthetic peptides, the presence of at least two putative heparin binding domains was shown, one at the N – terminus and another one, which is exposed on the opposite side and probably more accessible. Putative heparin or HS binding sites were further observed on

the ectodomains of the BMP receptors and HJV, strengthening the postulation that HS might act participate in the BMP6/BMPR/HJV complex formation (unpublished data).

While HS biosynthesis demands a great number of enzymes for the elongation and modification of the sugar chains, on the contrary, its degradation necessitates solely Heparanase – 1. The manipulation of its expression mirrors an avenue towards HS setting interference and towards the study of its effect on the hepcidin pathway.

4.3. The role of heparin in hepcidin regulation

Since the beginning of the past decade, evidence has come to light indicating that heparins strongly suppress hepcidin expression (Poli *et al.*, 2011). In hepatic HepG2 cells, hepcidin expression was found to be strongly suppressed by commercial heparins, both UFH (12 – 15 kDa) and LMWH (4.5 kDa), in a dose – dependent manner. This activity had a swift and evident occurrence upon 30 minutes, reached a peak at 4 hours and had a week – long duration or longer, in the presence of heparin. Mice treated with pharmacological doses of heparin displayed reduced hepatic hepcidin mRNA expression and SMAD phosphorylation, lower spleen iron reserves and elevated serum iron. In the same study, three hospitalized patients of old age received heparin for thrombosis prevention and were found to present lower serum hepcidin and higher serum iron levels (Poli *et al.*, 2011). The sole use of heparin as an inhibitor of hepcidin was obviously a subject not easily investigated due its anticoagulant property. A study of heparin chemistry would contribute to modifying the heparin molecule in order to reduce or delete the anticoagulant function. Heparin consists of disaccharide units, which contain one amino sugar, i.e. D-glucosamine, GlcN and one uronic acid, i.e. D-glucuronic acid, GlcA or L-iduronic acid, IdoA. The former is mostly N-sulfated or N-acetylated and 6-O-sulfated, whereas the latter is mainly sulfated at the position 2. Heparin's main structure consists of a 70% N-sulfated region (NS, IdoA2SO3-GlcNSO36SO3), N-acetylated region (NA, GlcA-GlcNAc) and mixed NA/NS (GlcA-GlcNSO3). The major alterations to diminish the anticoagulant activity are: 2O- and/or 6-O-desulfation, N-desulfation or N-acetylation, supersulfation or a simpler

reduction and oxidation of heparin, with the final product being the heparins termed as Glycol – split or RO-heparins (Casu, Naggi and Torri, 2002; Poli *et al.*, 2011; Poli, Asperti, Naggi, *et al.*, 2014; Poli, Asperti, Ruzzenenti, Regoni, *et al.*, 2014; Asperti *et al.*, 2015) (Table 3).

Table 3. List of the heparins studied for their anti – hepcidin activity in vitro and/or in vivo.

Compound	UFH (Commercial	LMWH	FONDAPARINUX	MH	R O – 82	R O – 68	NAc – 91	NAc – RO – 00	SSLMWH – 19
MW (kDa)	12-15	4.5	1.7	19.9	16	16.4	16	15.9	8.8
Anti-coagulant	+	+	+	+	-	-	-	-	+/-
Anti–hepcidin activity	Strong	Moderate	Low	Strong	Strong	Strong	Low	Low	Strong

FONDAPARINUX: Commercial pentasaccharide [Arixtra]; LMWH: Commercial low molecular weight Enoxaparin sodium [Clexane]; MH: Mucosal; MW: molecular weight; NAc-91: N-acetylated; NAc-RO-00: N-acetylated, glycol-split; RO-68: Glycol-split partially 20-desulfated; RO-82: Glycol-Split, Reduced Oxy; SSLMWH-19: Supersulfated low-molecular-weight; UFH: Mucosal, commercial unfractionated [Calciparin] (Casu, Naggi and Torri, 2002; Poli *et al.*, 2011; Poli, Asperti, Naggi, *et al.*, 2014; Poli, Asperti, Ruzzenenti, Regoni, *et al.*, 2014; Asperti *et al.*, 2015)

Glycol-split heparins are products of reduction and oxidation which induce glycol-bond collapse leading to structural modification of the ribbon, resulting in increased flexibility and a disrupted AT binding domain (Casu, Naggi and Torri, 2002). Both glycol-split heparins, coded RO-68 of 14 kDa molecular weight and RO-82 of 16.5 kDa, greatly suppressed hepcidin expression when administrated either alone or combined with stimulation of BMP6, both in primary hepatocytes and hepatoma cells (Poli, Asperti, Naggi, *et al.*, 2014). Upon subcutaneous administration in mice, those heparins lowered hepcidin levels within six hours with subsequent increase of serum iron and reduced spleen iron. They further suppressed hepcidin also following an acute stimulation of LPS and they improved recovery from anemia in mice inducted in anemia via a single injection of heat – killed Brucella abortus (Poli, Asperti, Naggi, *et al.*, 2014). Alternatively, heparin's anticoagulant function can be blunted by chemical introduction of additional sulfates (Toida *et al.*, 1999). A very low anticoagulant activity has been observed in both high and low molecular weight oversulfated heparins (Naggi *et al.*, 1987; Toida *et al.*, 1999), even though their other biological functions such as inhibition of cathepsin G and elastase are retained (Sissi *et al.*, 2006). More recently, the LMWH and highly sulfated heparin coded SSLMWH – 19 with a molecular weight of 8.8 kDa has been shown to prevent hepatic HepG2 cell hepcidin expression, with a concomitant significantly reduced spleen iron (Poli, Asperti, Ruzzenenti, Mandelli, *et al.*, 2014). This was further observed in an LPS treatment inflammation model and following a 10 – day heparin administration. A quicker and stronger activity in comparison with the Glycol – split heparins was noted; a potential explanation is that its lower molecular weight increases its bioavailability and the increased sulfation grade might facilitate electrostatic interactions (Weitz, 1997).

To the aim of testing the importance of the molecular weight and sulfate group density in heparins' anti – hepcidin activity, a study was conducted (Asperti *et al.*, 2015); Glycol-split RO – 82, partially desulfated Glycol-split RO – 68, oversulfated SSLMWH – 19 and mucosal heparin (MH) were isolated in fractions of different molecular weight and their anti – hepcidin function was investigated. The anti – hepcidin property reduced in accordance with the molecular weight and below 7-8 kDa, it was minimal, save the oversulfated SSLMWH – 19 heparin, which displayed a strong activity even below 4 kD, potentially thanks to its higher sulfated group

number. This data was recreated in mice, since unfractionated heparins displayed full inhibition at 40 mg/kg, whereas no inhibition was demonstrated in their 3.9 and 6.8 kDa fractions. An interesting finding was the evidence of hepcidin suppression even at 20 mg/kg of the oversulfated heparin of 4 kDa, suggesting that SSLMWH – 19 displays higher potency. The next anti – hepcidin activities to be tested were those of 2-O- and 6-O-desulfated heparin compounds, which turned out significantly reduced compared to the control heparin's both in HepG2 cells and mice, where almost no inhibition was noted. Additionally, a much lower binding affinity for a BMP6 synthetic peptide was noted when compared with unmodified control heparin, supporting the hypothesis that potency of the anti – hepcidin activity is also related to heparin's capacity to bind BMP6. Therefore, sulfation at positions 2-O and 6-O is important for the disruption of the hepcidin expression pathway. The observation that increased molecular weight and increased sulfation potentiate anti – hepcidin property indicates that heparin partakes in multiple binding sites that, besides involving BMP6, might also include BMP receptors and co-receptors to prevent the SMAD1/5/8 signaling.

As mentioned further above, heparin is a member of the GAG family its chemistry greatly resembles that of the endogenous heparan sulfates. Therefore, the observations indicate a role of liver heparan sulfate proteoglycans in regulation of hepcidin, which can be antagonized by exogenous heparin. Heparin and heparin sulfate are two terms often used interchangeably, albeit not correctly. Heparan sulfate occurs actually in all cells naturally and displays a wide variation both in its sulfation grade and in its molecular weight, ranging from 20 – 100 kDa, depending on its biological origin. Heparin derives from heparin sulfate as a product of degradation, isolated from equine lung or porcine entrails and its typical molecular weight range is 7 – 20 kDa. Heparin further displays the rare 3-O-sulfate groups, which are essential for its binding to AT, NS domains of great size and wide modification, with sizeable fractions of its chains consisting of IdoA and of fully sulfated (trisulfated) disaccharides, considerations stimulating a potential role of endogenous heparan sulfates also participating in the regulation of expression of liver hepcidin (Asperti *et al.*, 2015). Studies, which have been previously reviewed (Asperti *et al.*, 2019), have been conducted to verify this possible role that involved

the use of heparanase overexpression and preliminary data regarding alteration of heparan sulfate sulfation, but exceed the purpose of the present thesis.

ΕΙΔΙΚΟ ΜΕΡΟΣ

As stated previously, the strong anti – hepcidin activity of heparin, which has not been studied among humans, is heavily dependent on a high molecular weight, above 7 kDa (Asperti *et al.*, 2015). We thus hypothesized that unfractionated heparin, with a high molecular weight ranging from 12 to 15 kDa, would efficiently suppress expression of hepcidin amongst a sample of critically ill patients. Our choice to investigate the activity of unfractionated heparin is also attributed to further concerns regarding the adequacy of the bioavailability and systematic action of the anticoagulant in critically ill patients (Dörffler-Melly *et al.*, 2002; Priglinger *et al.*, 2003). To that end, we designed the present study analyzed in the present thesis, with a primary aim to verify to what grade – if at all – heparin reduces levels of hepcidin among critically ill patients being nursed in the ICU; to the best of our knowledge, this is the first human study to be conducted.

METHODS

1. Study design

This is prospective, non-invasive, observational study, conducted during the time period between October 2017 and December 2019, in a ten bed ICU. The primary aim of the study was the correlation of hepcidin levels with the administered unfractionated heparin in critically ill patients being hospitalized in the ICU. To that end, 22 patients nursed in the ICU were enrolled, after having acquired informed consent from themselves or their next of kin. Our study was approved by the Ethics Committee of our institution, General Thoracic Diseases Hospital of Athens "Sotiria". The prerequisite criteria for inclusion in the study was a hospitalization in the ICU of a duration lasting at least 5 days and administration of unfractionated heparin, prescribed solely at the attending physician's discretion for any etiology, either anticoagulant treatment or thromboprophylaxis. Patients to be excluded from the study included those in whom unfractionated heparin administration was ceased following the first 24 hours of hospitalization or those being subjected to Continuous Renal Replacement Therapy (CRRT). Blood sample analysis was conducted on four specific time points: once on admission, before the onset of heparin administration, considered as the baseline and also after the 1^{st}, 2^{nd} and the 5^{th} day of administration. In case of a patient having received LMWH, the first blood sample would be received more than 24 hours following the last LMWH dose. From any sample, serum and plasma was obtained through centrifugation and stored at $-80^{o}C$ up to the point of hepcidin level measurement. A further evaluation included iron metabolism parameters, particularly serum iron, ferritin and soluble transferrin receptors (sTFR).

The measurement of hepcidin levels was conducted through Quantikine Human Hepcidin Immunoassay via ELISA processing (R&D Systems, Minneapolis, MN, USA). Serum sTFR was determined through the Medilyzer Bx biochemical analyzer (MEDICON HELLAS S.A., Athens, Greece) by employment of the chemiluminescence method. Serum ferritin values were

calculated through the Alinity i Ferritin Immunoassay (Abbott Laboratories, Chicago, IL, USA), which is a chemiluminescent microparticle immunoassay.

At the same time, the included patients' demographics were recorded along with their coexisting diseases, clinical and laboratory parameters. The laboratory evaluation included: full blood count, baseline clotting (PT, aPTT, Fibrinogen, d-Dimers), basic biochemical parameters (urea, creatinine, electrolytes, bilirubin) and C-reactive protein (CRP). The Acute Physiology and Chronic Health Evaluation (APACHE) II (Knaus *et al.*, 1985) score at the time of admission and additionally, daily Sequential Organ Failure Assessment (SOFA) (Jones, Trzeciak and Kline, 2009) scores were calculated.

2. Statistical analysis

Categorical variables analysis was conducted through Fisher's exact test. Through Kolmogorov-Smirnov test, we evaluated the normality of continuous variables. Continuous variables at four separate time points (baseline, 24h, 48h 5d) were compared through a non-parametrical method, specifically Friedman's test. Variables that were measured repeatedly were entered in linear regression models fitted with generalized estimating equations (GEE), an extension of the generalized linear model that accounts for within-subject correlation. Time, a four-level ordinal variable (baseline, 24h, 48h 5d), was entered as within-subject correlation variable. The response variables' (hepcidin, ferritin, fibrinogen, sTFR, iron levels, d-Dimers, CRP) association with explanatory variables (heparin, creatinine, hemoglobin, e.t.c.) was modeled. Correction for age and SOFA score was entered in all models. In all GEE models an unstructured correlation structure was used and the Quasi Likelihood Information Criterion (QIC) was used for model selection. Data analysis was performed with SPSS 17.0 (2008; IBM Corporation, Armonk, NY, USA). Alpha was set at 0.05 (two-tailed) for all the analyses.

RESULTS

1. Demographic and Clinical Characteristics of the Study Group

The sample of the study was consisted of nine female and thirteen male patients, with a mean age (±SD) of 72.6±9.6 years and a BMI of 30.1±6.7 kg/m^2. The median ICU length of stay (ICU-LOS) was 13 (5.8 – 26,8) days (interquartile range). ICU mortality was 27.3 % (95% confidence interval 17.1 – 47.5) and the mean APACHE II score at the time of admission was 24.5 ±9.8. Out of the 22 enrolled patients, one patient was treated with heparin only for the first 96 hours and two were treated with heparin just for the first 48 hours. Out of 415 patients admitted in the ICU over the course of the study, only 22 patients were included (5.3%) mainly due to the fact that unfractionated heparin has been greatly replaced by LMWH as the anticoagulant of choice in ICUs, mostly because of its ten-times-less reduced risk for heparin induced thrombocytopenia (HIT) when compared to unfractionated heparin (Warkentin *et al.*, 1995, 2003, 2006). None of the included patients presented HIT. Sixteen patients were diagnosed with sepsis or septic shock, two patients with chronic obstructive pulmonary disease exacerbation, while the other diagnoses, one for each of the remaining four patients, were: idiopathic pulmonary fibrosis exacerbation, heart failure due to myocardial infarction, critical lower limb ischemia with circulatory collapse and polyserositis. In Table 4, the clinical and laboratory results of the included patient population are demonstrated.

Table 4. Clinical and laboratory characteristics of patients at ICU admission and upon 1st, 2nd and 5th day of treatment. (n = 22).*

	Variable	Admission	1st Day	2nd Day	5th Day	P value
Heparin & iron metabolism parameters	Heparin Dose (IU)	0	15115 ± 6691	18495 ± 11784	15790 ± 12875	<0.001
	Hepcidin (pg/ml)	27680 ± 12604	24100 ± 13930	22634 ± 13976	23382 ± 13706	0.284
	Iron (mg/dl)	28.9 ± 25.2	30.8 ± 23.1	29 ± 12.3	39.6 ± 24.2	0.081
	Ferritin (ng/ml)	705 ± 682	639 ± 686	708 ± 711	702 ± 674	0.874
	sTFR (mg/l)	2.00 ± 2.13	2.42 ± 4.06	1.75 ± 1.52	1.59 ± 1.05	0.644
Basic clinical & laboratory parameters	SOFA Score	7.50 ± 2.14	6.89 ± 1.84	6.44 ± 2.38	6.00 ± 3.14	0.705
	SaO_2 (%)	96 ± 2	96 ± 2	97 ± 2	97 ± 2	0.386
	PaO_2 (mmHg)	85 ± 11	90 ± 19	92 ± 18	89 ± 23	0.862
	$PaCO_2$ (mmHg)	42 ± 10	45 ± 9	45 ± 10	45 ± 9	0.860
	pH	7.40 ± 0.04	7.40 ± 0.06	7.41 ± 0.04	7.38 ± 0.08	0.957
	Lactate (mmol/l)	1.48 ± 0.83	1.24 ± 0.40	1.29 ± 0.81	1.77 ± 2.42	0.484
	Fibrinogen (mg/dl)	194 ± 178	189 ± 181	176 ± 181	234 ± 194	0.909
	d-Dimers (mg/l)	7.64 ± 8.13	6.99 ± 8.05	7.38 ± 9.06	6.63 ± 6.19	0.957
	Hemoglobin (g/dl)	11.7 ± 5.2	11.4 ± 4.9	11 ± 4.3	10.4 ± 3.8	0.004
	WBC (/µl)	13705 ± 4371	13236 ± 4163	12313 ± 3073	14215 ± 6683	0.671
	PLT (/µl)	279110 ± 1	283047 ± 1	280731 ± 1	260337 ± 1	0.185
	PT (sec.)	16.7 ± 2.9	16.6 ± 2.8	16.4 ± 3.2	18.3 ± 9.1	0.934
	aPTT (sec.)	41.9 ± 8.6	49.7 ± 11.6	51.2 ± 12.5	62 ± 20.5	<0.001
	INR	1.30 ± 0.27	1.29 ± 0.25	1.27 ± 0.29	1.52 ± 1.12	0.826
	CRP (mg/dl)	10.4 ± 7.3	10.4 ± 6.6	8.8 ± 5.9	9.3 ± 6.9	0.195
	Glucose (mg/dl)	176 ± 44	148 ± 41	176 ± 67	170 ± 77	0.056
	Urea (mg/dl)	98.3 ± 53.6	97.5 ± 45.5	95.4 ± 48.8	87.5 ± 31.5	0.868
	Creatinine (mg/dl)	1.42 ± 0.57	1.32 ± 0.52	1.36 ± 0.59	1.28 ± 0.52	0.053
	Albumin (g/dl)	2.87 ± 0.64	2.77 ± 0.61	2.68 ± 0.55	2.62 ± 0.62	0.575
	Bilirubin (mg/dl)	0.80 ± 0.64	0.72 ± 0.63	0.75 ± 0.56	0.74 ± 0.69	0.279
	Na^+ (mEq/l)	144 ± 6	145 ± 7	146 ± 6	145 ± 7	0.073
	K^+ (mEq/l)	4.20 ± 0.52	4.38 ± 0.45	4.20 ± 0.45	4.40 ± 0.49	0.498
	Ca^{2+} (mg/dl)	7.77 ± 0.55	7.78 ± 0.61	7.63 ± 0.52	7.72 ± 0.55	0.542
	Mg^{2+} (mEq/l)	2.25 ± 0.42	2.38 ± 0.37	2.40 ± 0.45	2.33 ± 0.39	0.592
	Cl^- (mmol/l)	106 ± 6	107 ± 7	109 ± 6	107 ± 9	0.012
	Pi (mg/dl)	3.61 ± 1.23	3.60 ± 0.81	3.81 ± 0.89	3.37 ± 0.81	0.602
	Insulin (IU)	26 ± 47	27 ± 53	23 ± 39	21 ± 33	0.889

*Values are mean ± SD

Abbreviations: SOFA: Sequential Organ Failure Assessment; SaO_2: hemoglobin oxygen saturation in arterial blood; PO_2: arterial partial oxygen pressure; $PaCO_2$: arterial partial carbon dioxide pressure; sTFR: soluble transferrin receptor; WBC: white blood cell count; PLT: platelets; PT: prothrombin time; aPTT: activated partial thromboplastin time; INR: international normalized ratio; CRP: C-reactive protein

2. *Analysis of the effect of Heparin on coagulation and inflammation parameters*

2.1. Repeated measures ANOVA

We applied repeated measures ANOVA with Greenhouse-Geisser correction. We adjusted consecutive models with the following independent variables:

1. Hepcidin
2. Ferritin
3. sTFR
4. Fibrinogen
5. d-Dimers
6. Serum Iron
7. CRP

In each model, the outcome (death/improvement), APACHE II score on admission and age were inserted as independent variables. In this analysis, a categorical variable is required for comparison between subjects. The variable utilized here was the outcome (two-tailed: survival/death). Sphericity check was conducted via Mauchly's test and for the analysis of dependent variables' fluctuation we used repeated measures ANOVA with Greenhouse-Geisser correction. All tests are two-tailed with significance level $\alpha=0.05$.

No significant difference was noticed in hepcidin, ferritin, sTFR, Fibrinogen, serum iron and CRP in comparison between the four time points (within subject) and between outcome possibilities (between subjects). All models were corrected for APACHE II score on admission and age.

In the d-Dimers variable, there was a significant difference between survivors and deceased patients (between subjects) which concerned time points 2 (24h) and 3 (48h). Specifically, based on the adjusted models, deceased patients had an 11.5 higher (95% confidence interval

3.38 – 19.61, p=0.009) mean value of d-Dimers at 24h than survivors; similarly, at 48h this difference was 12.36 (95% confidence interval 2.87 – 21.85, p=0.015). The above models are corrected for APACHE II score on admission and age. The d-Dimers values upon the 4 time points in regard with the outcome are presented in figure 4.

Figure 4. Comparison of d-Dimers levels between surviving and deceased patients.

If time point 4 is excluded from the analysis, i.e. the 5th day and only the first three time points are included, in the resulting repeated measures ANOVA model, there is a significant within subject difference in d-Dimers value in-between the time points (p=0.003). A similar significance is noticed in the interaction between: a) time and APACHE II score (p<0.001) and b) time and outcome (p=0.003). The interpretation of these interactions is that, apart from the significant difference existing between the time points, there is a further effect on this

difference depending on APACHE II score and outcome. There is also a trend towards interaction with age.

In the above 3-time point analysis, based on the adjusted models, mean d-Dimers value at 24h was higher by 11.8 (95% confidence interval 4.5 – 19.1, p=0.003) in deceased patients compared to survivors. Similarly, mean d-Dimers count upon 48h was higher by 12.5 (95% confidence interval 3.8 – 21.3, p=0.008) among deceased patients versus survivors. The above models are corrected for APACHE II score on admission and age. Of note, fluctuation of d-Dimers count through time is evaluated by a multivariate model of analysis in which, as previously mentioned, APACHE II score, age and outcome have been imported as explanatory variables.

2.2. General estimating equation (GEE) - Linear models

Linear regression models were adjusted with the aforementioned (1-6) dependent variables. Time (i.e. time point of measure) was used as within-subject variable. Therefore observations of the same subjects on separate time points were examined by the models as connected with consecutive succession that is based on the variable "time" (1=baseline, 2=24h, 3=48h, 4=5d). As independent variables, we defined SOFA score of each time point, age, heparin dose and outcome.

An advantage of this type of analysis is the possibility of introduction of heparin dose at each time point as an independent variable and examination of the correlation of dosage with a dependent variable, with a correction for clinical state gravity (SOFA score at separate time points), age and outcome. A drawback is that the emerging evaluation concerns an overall effect of heparin and the impossibility to detect neither the time point nor the dose at which said effect takes place.

The B coefficient for each variable represents the model's evaluated alteration of the variable that results from a 1000 IU increase of the administered heparin dose:

Independent variable*	B coef.	p value	95% confidence interval
Hepcidin (pg/ml)	-542.6	<0.001	-699.9 to -385.3
Serum Iron (mg/dl)	0.129	0.530	-0.273 to 0.530
Ferritin (ng/ml)	-13.9	<0.001	-21.0 to -6.8
sTFR (mg/l)	0.012	0.084	-0.002 to 0.025
CRP (mg/dl)	-0.11	0.030	-0.210 to -0.011
Fibrinogen (mg/dl)	-3.95	0.001	-6.18 to -1.72
d-Dimers (mg/l)	-0.129	0.016	-0.233 to -0.024
Lactate (mmol/l)	-0.002	0.717	-0.011 to 0.007

*Linear models GEE with heparin dose, age, SOFA score and outcome as independent variables.

Accordingly, without defining outcome as an independent variable, we get the following results:

Independent variable	B coef.	p value	95% confidence interval
Hepcidin (pg/ml)	-533.1	<0.001	-706.8 to -357.5
Serum Iron (mg/dl)	0.114	0.570	-0.279 to 0.507
Ferritin (ng/ml)	-13.9	<0.001	-21.4 to -6.4
sTFR (mg/l)	0.015	0.071	-0.001 to 0.030
CRP (mg/dl)	-0.102	0.036	-0.197 to -0.007
Fibrinogen (mg/dl)	-3.65	0.003	-6.07 to -1.24
d-Dimers (mg/l)	-0.156	0.001	-0.246 to -0.066
Lactate (mmol/l)	-0.002	0.686	-0.011 to 0.007

As observed, the differences in evaluated parameters are small. A noteworthy difference is in the evaluation of beta coefficient for d-Dimers. As mentioned previously, d-Dimers values differ significantly between groups of different outcomes (at time points 2 and 3). We notice that in the second model, not including the "outcome" variable in the analysis, the estimated beta coefficient for heparin is greater (-0.156 versus -0.129, a more negative correlation) and p value is lower (0.001 versus 0.016). This discrepancy is recorded because the model is not corrected for outcome.

In a second analysis, we adjusted linear models GEE, this time by defining hepcidin as a dependent variable and the following independent variables:

1. Heparin
2. Age
3. SOFA score
4. Creatinine
5. Hemoglobin
6. CRP
7. PaO_2
8. Serum Iron

Import of variables heparin, age, SOFA score was mandatory in all models. The variables norepinephrine, creatinine and hemoglobin were initially imported separately along with the "mandatory" variables [heparin], [age], [SOFA score] and subsequently in combinations, for the variables that showed a significant effect in previous models.

We hereby present the following models in which significant effects were noticed. All models are corrected for SOFA score and age.

1.

Independent variables	B coef.	p value	95% confidence interval
Heparin (per 1000 IU increase)	-408,6	<0,001	-572,7 to -244,4
Creatinine (per mg/dl increase)	4782,7	0,004	1527,3 to 8038,2

2.

Independent variables	B coef.	p value	95% confidence interval
Heparin (per 1000 IU increase)	-420,0	<0,001	-583,5 to -256,6
Creatinine (per mg/dl increase)	4401,2	0,016	831,2 to 7971,2
Hemoglobin (per mg/dl increase)	-317,5	0,258	-868,1 to 233,1

3.

Independent variables	B coef.	p value	95% confidence interval
Heparin (per 1000 IU increase)	-375,1	<0,001	-553,4 to -196,9
Creatinine (per mg/dl increase)	3645,2	0,032	306,1 to 6984,4
CRP (per mg/dl increase)	518,5	0,085	-71,33 to 1108,4
pO_2 (per mmHg increase)	-65,5	0,237	-174,1 to 43,2

4.

Independent variables	B coef.	p value	95% confidence interval
Heparin (per 1000 IU increase)	-376,6	<0,001	-557,0 to -196,1
Creatinine (per g/dl increase)	4124,8	0,021	609,6 to 7640,0
CRP (per mg/dl increase)	468,8	0,108	-102,5 to 1040,2

5.

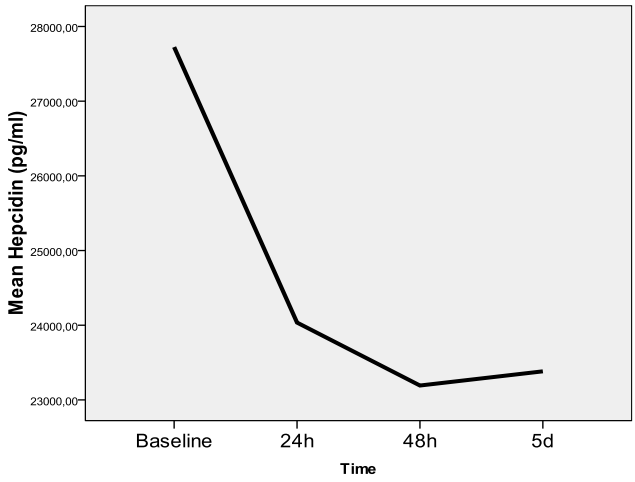
Independent variables	B coef.	p value	95% confidence interval
Heparin (per 1000 IU increase)	-538,9	<0,001	-717,6 to -360,2
Fe (per mg/dl increase)	65,7	0,477	-115,4 to 246,9

Heparin displayed a strong independent negative association with hepcidin, and this effect was robust, persisting in all fitted models with a high statistical significance ($p<0.001$). Beta coefficients of heparin in different linear models ranged between -539 and -375, meaning that an estimated decrease of hepcidin levels by 375 to 539 pg/ml is expected for every 1000 IU increase in heparin dose administered.

Of other explanatory variables tested, creatinine displayed an independent positive association with hepcidin with beta coefficients in different models ranging between 3645 and 4783, meaning that an estimated increase of hepcidin levels by 3645 to 4783 pg/ml is expected for every 1 mg/dl increase in creatinine levels. Iron did not present a significant correlation with hepcidin.

A brief summary of our study's results is demonstrated in Figure 5.

A. Hepcidin change over time.



B. Multiple regression model for hepcidin (n=22).

Independent variales*	B coef.	p value	95% confidence interval
Heparin (per 1000 IU increase)	-376,6	<0,001	-557,0 to -196,1
Creatinine (per mg/dl increase)	4124,8	0,021	609,6 to 7640,0
CRP (per mg/dl increase)	468,8	0,108	-102,5 to 1040,2

C. Beta coefficients for heparin (explanatory variable) in fitted linear models

Dependent variables*	B coef.	p value	95% confidence interval
Hepcidin (pg/ml)	-533.1	<0.001	-706.8 to -357.5
Serum Iron (mg/dl)	0.114	0.570	-0.279 to 0.507
Ferritin (ng/ml)	-13.9	<0.001	-21.4 to -6.4
sTFR (mg/l)	0.015	0.071	-0.001 to 0.030
CRP (mg/dl)	-0.102	0.036	-0.197 to -0.007
Fibrinogen (mg/dl)	-3.65	0.003	-6.07 to 1.24
d-Dimers (mg/l)	-0.156	0.001	-0.246 to -0.066
Lactate (mmol/l)	-0.002	0.686	-0.011 to 0.007

Figure 5. Study results.

Panel A illustrates the decrease of serum hepcidin levels during 5 days of intravenous administration of unfractionated heparin.
Panel B displays heparin's strong independent negative association with hepcidin, which persisted in all fitted models with a high statistical significance ($p<0.001$). Beta coefficients of heparin in different linear models ranged between -539 and -375, meaning that an estimated decrease of hepcidin levels by 375 to 539 pg/ml is expected for every 1000 IU increase in heparin dose administered. Creatinine displayed an independent positive association with hepcidin with beta coefficients in different models ranging between 3645 and 4783, meaning that an estimated increase of hepcidin levels by 3645 to 4783 pg/ml is expected for every 1 mg/dl increase in creatinine .levels. Of other explanatory variables tested, hemoglobin, serum iron, PaO_2 and CRP did not display a significant effect on hepcidin, although CRP did indicate a positive trend.
Panel C shows the estimated change in each parameter that results from a 1000 IU increase in the administrated heparin dose.
* All models are corrected for SOFA score and age.

CRP: C-reactive protein; sTFR: soluble transferrin receptors; PaO_2: partial oxygen pressure in arterial blood

DISCUSSION

The present study showed a significant reduction in hepcidin concentration by administration of unfractionated heparin to critically ill patients in the ICU. As far as we know, this is the first study in humans to test the action of unfractionated heparin on hepcidin production. It is also the first study performed on ICU patients to test the effect of any heparin on hepcidin production. This is especially important since, in these patients, many factors accumulate that can variably affect the metabolic pathway of the production of this molecule which plays a central role in iron metabolism; even more so, since a pleiotropic effect of heparins has recently been recognized, in addition to their anticoagulant properties (Torri and Cassinelli, 2018).

Factors that potentially affect hepcidin synthesis are inflammation (Jairam *et al.*, 2010; Małyszko *et al.*, 2012; Łukaszyk *et al.*, 2015), erythropoietic activity (Nemeth and Ganz, 2006) and iron deficiency/overload (Ramsay *et al.*, 2009; Hentze *et al.*, 2010).

In severely ill patients, septic or not, a generalized inflammatory condition usually occurs, the severity of which is indicated by CRP (Pepys and Baltz, 1983; Gabay and Kushner, 1999; Póvoa, 2002). Inflammation induces the synthesis of hepcidin. In addition, these patients often develop acute kidney injury (Chertow *et al.*, 2005), a condition in which hepcidin levels also appear to increase (Prowle *et al.*, 2012). In agreement with the above, in the patients studied, hepcidin showed a significant positive correlation with serum creatinine levels, while there was a trend for a positive correlation with CRP values, which, however, did not reach statistically significant levels.

Anemia and hypoxia are frequently observed in critically ill patients (Weiss and Goodnough, 2005; Kassebaum *et al.*, 2014). These constitute potential stimuli to activate erythropoiesis, a condition that represses hepcidin production. However, it should be noted that: 1. an inflammatory response, sepsis-induced or otherwise, that affects the respiratory system, may constitute severe hypoxia, e.g. in severe ARDS cases. As referred though, inflammation is in itself a stimulant for hepcidin production, and 2. in the development of anemia of critical illness may contribute both a blunted erythropoietin synthesis with impaired proliferation of erythroid

cells, resulting from inflammation and an iron deficiency from overt or occult blood losses; similarly, these conditions have an opposing effect on hepcidin production. In addition, in inflammatory conditions, serum iron and ferritin concentrations are not reliable indicators of iron stores (Suchdev *et al.*, 2017). For example, low iron and high ferritin are indicative of the inflammatory iron profile: hepcidin induced during inflammation may lead to decreased iron levels in blood while synthesis of ferritin increases in inflammatory conditions, regardless of iron stores. In our study, serum iron, hemoglobin and PO_2 did not show a significant correlation with hepcidin levels, in agreement, somehow, with the lack of an absolute, one-way causality interaction between the above factors and the production of hepcidin in the critically ill.

Another factor that may be implicated with hepcidin production could be gender; chronic liver diseases associated with reduced hepcidin expression include alcoholic liver disease, chronic hepatitis C and genetic hemochromatosis, all conditions that have been shown to demonstrate significant disparities in their distribution among genders (Harrison-Findik, 2010). Studies on mice have shown hepcidin inducement after castration in males and hepcidin reduction after daily administration of testosterone to females (Guo *et al.*, 2013; Latour *et al.*, 2014), while also notable is the possibility of implication of hepcidin that was demonstrated in a model of mice under chronic inflammation, where administration of testosterone to females reversed anemia (Guo *et al.*, 2016).

Despite the plethora of pathological conditions that would interfere with heparin action in inhibiting hepcidin production in critically ill patients, we should emphasize the persistent, strong association of heparin administration with the reduction of hepcidin concentration, regardless of any “confounder”. Arguably the action of heparin predominates over any other stimulus in the metabolic pathway that controls the production of this molecule since heparin inhibits hepcidin transcription, by binding BMP (Wozney *et al.*, 1988); the BMP pathway plays a pivotal role in hepcidin transcription and therefore, by sequestering BMP ligands, heparin attacks its production at a very early stage.

The results of the study also show that the administration of unfractionated heparin is accompanied by a reduction in CRP and ferritin levels, possibly indicating its anti-inflammatory

effect. We also noted a trend of increase in sTFR concentration by administering heparin. sTFR has been identified as a marker of erythropoiesis activation (Lorenzo *et al.*, 2001; Chiang *et al.*, 2002; Tarng and Huang, 2002; Braga *et al.*, 2014). Since it is known that hepcidin, by inhibiting the absorption of iron in the duodenum and preventing the recycling of iron by macrophages, ultimately leads to iron-restricted erythropoiesis (Camaschella and Nai, 2016), by translating this result in light of the current literature we could be led to assume that progressive restoration of erythropoietic activity may possibly ensue after treatment with heparin due to hepcidin reduction.

Finally, in addition to its anticoagulant effect, which is confirmed by an increase in aPTT levels, in our study heparin administration was also associated with a reduction in d-Dimers and fibrinogen, all in line with current bibliographic data (Ruggiero *et al.*, 1983; Speiser *et al.*, 1990; Raut and Gaffney, 1996; Minnema *et al.*, 1997).

Admittedly, hepcidin repression is not always a desirable situation. The resulting upregulation of ferroportin leads to export of iron to the circulation. Iron catalyzes the Fenton reaction (Winterbourn, 1995), i.e. the conversion of hydrogen peroxide to free radical ions, ultimately leading to oxidative stress. Catalytic iron has been found to be a key sentinel of acute kidney injury in animal models (Paller, 1988; Walker and Shah, 1988; Baliga, Ueda and Shah, 1993; Baliga *et al.*, 1996, 1998), while hepcidin seems to possess renoprotective functions (Scindia *et al.*, 2015; van Swelm *et al.*, 2016). Indeed, very recently, a large cohort study (Leaf *et al.*, 2019) of critically ill patients with acute kidney injury requiring renal replacement therapy associated higher plasma concentrations of catalytic iron and lower concentrations of hepcidin with increased mortality, suggesting the possible use of these parameters as prognostic markers. Furthermore, hepcidin promoter activity and hepcidin transcription in the liver is also inhibited by alcohol – induced oxidative stress (Harrison-Findik *et al.*, 2006) leading to the assumption that hepcidin could be a potential risk factor in the progression of alcoholic liver disease (Harrison-Findik, 2010). Obviously, “fine tuning” of the human metabolism through external interventions is rather difficult, it is essential, however, to be fully aware of said interventions’ full range of effects (therapeutic or side – effects).

Additionally, heparin's related effects are similarly not always positive; heparin chains, especially the longer ones, anticoagulant or not, bind strongly to molecules with a positive charge such as platelet factor 4 (PF4) and form a neoantigen complex which induces antigen – antibody reaction; the immune complex binds to Fc – gamma RII receptors (CD32), specifically on ECs and platelets, leading to their activation and destruction. This is the explanation for the thrombogenic and thrombocytopenic phenomena caused by heparin during type 2 HIT. This course of action and the neoantigen type translate into the immunological memory being erased after the course of several months and, following six months after HIT, the risk of recurrent HIT upon re-administration of heparin returns to the same as an unaffected individual's. That certainly however does not mean the danger is reduced shortly after a HIT event; far from it, the risk remains high and with potentially serious consequences in the first weeks and months past the initial HIT occurrence. As stated, the risk increases proportionately with the length of the chains and, therefore, it is absent with fondaparinux (Schindewolf *et al.*, 2017). The mechanism of action further indicates a potential cross – reaction with daparinoid, which may not consist of heparin but does consist of heparan sulphate, chondroitin sulphate and dermatan sulphate, with which specific similarly – configured chains may cross – react (Ronchard *et al.*, 2017).

Heparin's role is constantly evolving as novel indications and possibilities are discovered. The volume of literature regarding this issue merits the conduction of systematic reviews examining the variable applications, as for example the treatment of malignancies, streamlining birth rates in females with thrombophilia, utilization in percutaneous coronary procedures or in patients that are subjected to hemodialysis.

Certainly, despite the innumerous amount of publications, several issues still remain to be examined. In a recent systematic review and meta – analysis (Mousavi *et al.*, 2015), the administration of heparin and heparin byproducts was associated with improved outcomes among patients subjected to cardiopulmonary bypass, cataract surgery and asthma sufferers. However, studies evaluating heparin in patients diagnosed with ulcerative colitis were

characterized as heterogeneous and incompatible, thus inhibiting the drawing of concrete conclusions.

Anti – cancer treatments have always been a tempting subject to test agents developed to counter alternative diseases and this led to the identification of a considerable number of current treatment modalities. Gemcitabine, originally designed for viral disease treatment is currently one of the drugs most widely used for pancreatic cancer treatment. Heparin has been assessed among patients with variable cancer types, of different staging, with or without established VTE. In spite of this, systematic reviews still provide conflicting results; recently, an update of a systematic review (Lazo-Langner *et al.*, 2007) conducted by Sanford et al (Sanford *et al.*, 2014) drew opposite results compared to the previous one: even though the 2007 analysis suggested an improvement of survival rates in the patient group treated with LMWH (OR 0.70; 95 % CI 0.49–1.00, p = 0.05), the update of this analysis achieved no such result (OR 0.87, 95 % CI 0.70–1.08, p = 0.21).

A most recent study that especially deserves mention is the meta – analysis by Fu et al. (Fu *et al.*, 2022) which included 15 RCTs with a total of 2617 patients and evaluated the efficacy of UFH in an adult patient population with sepsis; the authors observed that UFH was associated with a reduced 28-day mortality rate (RR: 0.82; 95% CI: 0.72 to 0.94, $p < 0.05$), even more so for patients with an Acute Physiology and Chronic Health Evaluation II (APACHE II) score greater than 15 (RR: 0.83; 95% CI: 0.72 to 0.96). More specifically, the 28-day mortality was relatively reduced by 16% among patients treated with UFH, whilst among sepsis patients with an APACHE II score higher than 15, 28-day mortality rate relative reduction reached a percentage of 17%. Additionally, UFH led to a lower multiple organ dysfunction syndrome (MODS) incidence (RR: 0.61; 95% CI: 0.45 to 0.84, p = 0.002), length of stay in the ICU (MD: -4.94; 95% CI: − 6.89 to − 2.99, $p < 0.00001$) and duration of mechanical ventilation (MD: -3.01; 95% CI: − 4.0 to − 2.02, $P < 0.00001$). Not only did UFH demonstrate no impact upon adverse bleeding events (RR: 1.10; 95% CI: 0.54 to 2.23, p = 0.80), but actually in the UFH treated group laboratory coagulation values were improved as well since, the platelet count was higher (MD: 9.18; 95% CI: 0.68 to 17.68, p = 0.03) and the activated partial thromboplastin time (APTT) was

shorter (MD: -8.01; 95% CI: − 13.84 to − 2.18, p = 0.007); PT results failed to demonstrate any statistically significant difference (MD = − 0.05; 95% CI = − 1.34 to 1.23; P = 0.93 > 0.05; I2= 81%), a considerable heterogeneity however was an issue, according to the authors. As such, the most recent evidence indicates that UFH may lead to improve the clinical efficacy amongst sepsis patients without increasing adverse bleeding risks and, in doing so, improve 28-day mortality.

LIMITATIONS

The present study has some limitations. Due to its small sample (n=22), our study was underpowered to detect a significant effect of C-reactive protein on hepcidin, even though a positive trend was found. Another limitation is the lack of a matched control group. However, ICU patients requiring thromboprophylaxis could not – actually it would be unethical according to international guidelines – be randomized into a group not receiving anticoagulant treatment without specific contraindications dictating its avoidance, e.g. low platelet count, major bleeding, e.t.c. Moreover, a cohort of patients with contraindications to anticoagulants would definitely not be matched with patients who could receive anticoagulant treatment and therefore they would necessarily differ. The same applies to patients receiving treatment or prophylaxis with low molecular weight heparins, which are generally preferred over unfractionated heparin, the administration of which follows certain criteria that necessarily differentiate patients receiving it from the rest thus creating a serious sampling bias, for instance application of different criteria for therapy allocation. Nevertheless, we propose that the findings of the present study would generate a rationale to spark future RCTs or well-designed nested case–control studies, which would safely draw conclusions concerning the above-mentioned comparison.

STRONG POINTS – FUTURE RESEARCH PROPOSALS

Our study also has some strong points; as mentioned above, we have presented a robust correlation between heparin and hepcidin - that is not toppled by the aforementioned confounders; we also managed to quantify said correlation. In addition, to the best of our knowledge, this is the first study with a sample consisting solely of humans, and critically ill patients for that matter, who present with several pathologies that may be implicated as "confounders" in the expression of hepcidin, and we hope that our study can serve as footing for more studies on humans in the future. Further studies should aim for establishment of study designs that include a greater number of patients enrolled or organizing blinding methods, therefore minimizing bias in order to reach concrete conclusions, cementing the role of heparin as a hepcidin repressor among critically ill patients. Furthermore, it would be advisable to systematically review the variable applications of heparin, which through the hundred years of its discovery, has shown that it is more than just an anticoagulant.

Even though not all conclusions regarding the real effect of heparin on the several conditions discussed in the present thesis have been cemented beyond any doubt, research will most definitely continue in a steadfast pace, as novel horizons draw nearer. Studies evaluating the use of heparin on tissue engineering, stem cell therapies and regenerative medicine are already underway, ensuring heparin's spotlight in medicine for the following decades.

CONCLUSION

Treatment of critically ill patients with heparin has displayed contradictory results over the years. One thing that is for certain is that description of heparin as an anticoagulant is an outdated characterization; besides anticoagulant properties, heparin also exhibits anti-inflammatory, glycocalyx-protective and anti-hepcidin functions, which add to the pleiotropic effect of heparin that has recently been under constant scrutiny. Thromboinflammation, Iron Refractory Iron Deficiency Anemia and Anemia of Inflammation are pathologic conditions which are greatly common amongst critically ill patients, therefore the aforementioned properties of heparin are very likely to constitute this agent as an effective therapeutic treatment in the future. So far this potential role remains to be elucidated and for that reason, future research should focus on establishing study designs that are potent to minimize bias and reach concrete conclusions. Hopefully, heparin may play an essential role in reducing the severity of sepsis, ameliorating anemia or mitigating its complications, shortening the duration of hospitalization and its accompanying costs due to it and, above all, in improving the still unsatisfactory survival rates of critically ill patients.

The century – old history of heparins might lead someone to presume that an “ancient” drug such as this would be victimized by the pharmacological modernity. The evolution of medicine mandates the development of newer modalities that undoubtedly provide alternatives to prevention and management of VTE and will be more than welcome. However, the complexity and pleiotropic effects of said glycosaminoglycans still challenge the scientific community, to the point at which previous approaches strangely emerge repeatedly in medical literature. All the new indications, next to all those cemented long ago, topple the image of an outdated medication and give birth to high expectations for the near future.

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