Heparin: an established anticoagulant drug with therapeutic potential for COVID-19

Master of Philosophy Biomedical Science

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Abstract:

The biologically and medically important heparan sulphate and heparin polysaccharides have been previously shown to mediate viral cell entry for enveloped viruses. Furthermore, heparin and its analogues have been shown to have anti-inflammatory and anti-coagulation activity, this presents an opportunity to develop a multipronged therapeutic agent that can be used to mediate not only viral activity *in vivo*, but the often-accompanying inflammation and coagulopathies too. Research groups investigating the activity of heparin and its analogues have long demonstrated that heparin can inhibit a broad range of enveloped viruses, however, research regarding the novel SARS-CoV-2 is still ongoing.

In this study, a scoping review of the current and developing research regarding heparin and SARS-CoV-2 was carried out to determine that heparin and heparin analogues not only bound SARS-CoV-2 receptor binding domain but modulated viral activity. Further investigation to determine the optimal form of heparin or type of heparin analogue revealed that low molecular weight heparins, heparin mimetics and sulphated plant compounds can exert similar, in some cases superior, viral binding inhibition when compared to standard unfractionated heparin. Of the research available, it was demonstrated that prophylactic or therapeutic heparin is associated with a reduced mortality risk, compared to other standard treatments. Despite promising results there is not an abundance of available research regarding heparin as a potential therapeutic for SARS-CoV-2 limiting the scope of the review.

Differential scanning fluorimetry was utilised to observe the thermal destabilisation of SARS-CoV-2 receptor binding domain in the presence and absence of heparin. This demonstrated that heparin destabilised the secondary protein structure of the SARS-CoV-2 receptor binding domain in two different mutations, UK variant and GenBank: MN908947, by 2.59°C and 2°C respectively. Investigation using an enzyme linked immunosorbent assay determined the presence of heparin inhibited the binding of SARS-CoV-2 receptor binding domain to one of its primary entry molecules, the ACE2 receptor, by 42%. Finally, a library of variably sulphated plant compounds was used to identify SARS-CoV-2 receptor binding domain inhibitors, with beneficial therapeutic attributes including low molecular weight and reduced off-target effects. Of the 29 sulphated plant compounds investigated, all had inhibitory activity on the binding activity of SARS-CoV-2 RBD to ACE2 receptors, 17 of which were more inhibitory than heparin, the most potent were HSer-C, FVes-F, HS-F and SL-K. This provides a basis of evidence that the approved drug heparin has pleiotropic effects beneficial for a disease that progresses to impact several organ systems, with multi-therapeutic effects. But may elucidate the structural requirements for the inhibitory activity displayed, which could be used to more specifically tailor a form of heparin or heparin analogue that is both effective and has minimal off-target and adverse effects.

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List of Abbreviations:

+ssRNA: Positive-single-stranded RNA

ACE2: Angiotensin-converting enzyme 2

AHRF: Acute hypoxemic respiratory failure

APN: Aminopeptidase N

aPTT: Activated partial thromboplastin time

AT: Antithrombin

ATIII: Antithrombin

BCoV: Bovine coronavirus

CCoV: Canine coronavirus

CD: Cluster differentiation

CHO cells: Chinese hamster ovary cells

CNS: Central nervous system

CoVs: Coronaviruses

CS: Chondroitin sulphate

DAMP: Damage-associated molecular pattern

DPP4: Dipeptidyl peptidase 4

DS: Dermatan sulphate

ECM: Extracellular membrane

EGF: Epidermal growth factor

ER: Endoplasmic reticulum

ERGIC: Endoplasmic reticulum-Golgi intermediate compartment

ExoN: Exoribonuclease

EXT: Exostosin

FGF: Fibroblast growth factor

FIPV: Feline infectious peritonitis virus

FXa: Factor Xa

GAG: Glycosaminoglycan

Gal: Galactose

GalNAc: *N*-acetyl-β-D-galactosamine

GlcA: Glucuronic acid

GPI: Glycosylphospatidylinositol-anchored

HA: Hyaluronic acid

HBV: Hepatitis B virus

HCoV: Human coronavirus

HCV: Hepatitis C virus

HE: Haemagluttin-esterase

HGF: Hepatocyte growth factor

HIT: Heparin-induced thrombocytopenia

HIV: Human immunodeficiency virus

HSPE: Heparanase

ICAM: Intracellular adhesion molecule

IdoA: Iduronic acid

IFN: Interferon

IRFs: IFN regulatory factors

JAK: Janus kinase

KS: Keratan sulphate

LMWH: Low molecular weight heparin

M: Membrane

mCEACAM: Murine carcinoembryonic antigen-related adhesion molecule

MCP: Monocyte chemoattractant protein

MDA5: Melanoma differentiation associated protein

MERS-CoV: Middle East respiratory syndrome coronavirus

MH: Modified heparin

MHV: Murine hepatitis virus

MIP: Macrophage inflammatory protein

MMP: Metalloproteinases

Mpro: Main protease

MtDNA: Mitochondrial DNA

NACH: Non-anticoagulant low molecular weight

NendoU: Viral endoribonuclease, 2′-O-MT: 2′-O-methyltransferase

NET: Neutrophil extracellular trap

NG2: Nerve glial antigen 2

Nsp: Non-structural proteins

ORFs: Open reading frames

PAMP: Pathogen-associated molecular pattern

PCS: Prospective cohort study

PDGF: Platelet-derived growth factor

PEDV: Porcine epidemic diarrhoea virus

PF4: Platelet factor 4

PG: Proteoglycan

PGSL-1: P selectin glycoprotein ligand

PLA: Platelet-leucocyte aggregate

PLpro: Papain-like proteases

PP: Polyproteins

R_0: Reproductive number

RABV: Rabies virus

RBD: Receptor binding domain

RCL: Reactive centre loop

RCS: Retrospective cohort study

RCT: Randomised control study

RdRp: RNA dependent RNA polymerase

RTC: Replicase-transcriptase complex

SARS: Severe acute respiratory distress syndrome

SARS-CoV: Severe acute respiratory syndrome coronavirus

SF: Scatter factor

SGH: Split glycol heparin

SIC: Sepsis induced coagulopathy

SLRPs: Small leucine rich proteoglycans

SPR: Surface plasmon resonance

STAT1: Signal Transducer and Activator Of Transcription 1

TGEV: Transmissible gastroenteritis virus

TLRs: Toll like receptors

TMPRSS2: Transmembrane protease serine 2

TNF-α: Tumour necrosis factor-α

tPA: Tissue plasminogen activator

TSR: Transcriptional regulatory sequences

TxA2: Thromboxane A2

UA: Uronic acid

UFH: Unfractionated heparin

UTR: Untranslated region

VEGF: Vascular endothelial growth factor

VTE: Venous thromboembolism

Chapter 1: Introduction

1. Glycosaminoglycans and proteoglycans:

1.1. Proteoglycans

Proteoglycans (PGs) are a heterogenous class of biomolecules that are multifaceted in nature and have the common feature of a protein core with covalently bound projecting glycosaminoglycan (GAG) chains (Couchman & Pataki, 2012). Glycosaminoglycans are a structurally diverse class of polyanionic, linear carbohydrate polymers, comprised of repeating disaccharide units with alternating monosaccharide residues (Gandhi & Mancera, 2008). The core proteins of known proteoglycans vary vastly in size from 11,000 to approximately 220,000 Daltons (Bertolami & Messadi, 1994). Proteoglycans are a ubiquitous group of molecules with varied actions as a result of the variation in GAG chains that can attach, the polydisperse nature of their binding and the wide variety of protein cores to which GAGs can attach (Theocharis, *et al.,* 2008).

The GAG family consists of chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), hyaluronan or hyaluronic acid (HA), heparan sulphate (HS) and heparin (Chakrabarti & Park, 2008). The disaccharide units of GAGs alternate between hexosamine and uronic acid (UA), with the exception of keratan sulphate, resulting in a structurally complex polysaccharide backbone (Tumova, *et al*., 2000). The UA units can be β-D-glucuronic acid (GlcA) or its C_5 epimer, α-L-iduronic acid (IdoA) (Lindahl, 1999). These subunits can vary in amino sugar composition, glucosamine or galactosamine, and amino group substitution and amino group substitution, hexosamines can be N-sulphated, N-acetylated or lack substitution all together (Gandhi & Mancera, 2008). Keratan sulphate, however, is composed of repeating disaccharide units of galactose (Gal) and N-acetylglucosamine (Hoshino, *et al.,* 2014). High structural variability of glycosaminoglycans is a result of permutations of the monosaccharides, modifications to saccharide unit positions, length of chain and varying degrees and position of sulphation, which is specific to cell origin and environment (Kjellen & Lindahl, 1991) (Hagner-McWhirter, *et al.,* 2004).

As a result of this structural diversity PGs have a multitude of biological functions conferred by binding sites for multiple different ligands (Hardingham & Fosang, 1992). Proteoglycans can be classified into three categories characterised by the assembly of various protein modules: cell surface proteoglycans, modular proteoglycans, and small leucine rich proteoglycans (SLRPs) (Schaefer & Schaefer, 2010). Modular PGs permit the assembly of protein modules into a highly glycosylated and elongated single structure (Iozzo & Murdoch, 1996) (Schaefer, 2014). This group can be further subdivided into HA-binding PGs known as hyalectans (including aggrecans, brevican, neurocan and versican) and non-hyaluronan binding PGs (including agrin, collagen XVIII and perlecan) (Gaudet & Popovich, 2014). SLRPs are characterised by small leucine-rich-repeats in the primary structure (Iozzo, 1997) and the presence of N-terminal Cys clusters, 4 cysteine residues with finite intervening amino acid sequences (Schaefer & Iozzo, 2008). This group can be subdivided into 5 distinct classes based on conservation and homology at genomic and protein

levels, chromosomal organisation, and the presence of N terminal-Cys clusters (Nastase, *et al.,* 2014). Most research of SLRPs has been directed towARDS Class 1, which includes asporin, biglycan and decorin, of which biglycan and decorin have been the most explored (Pietraszek-Gremplewicz, *et al.,* 2019). The third group, cell surface PGs, are divided into glypicans and syndecans, serglycin, however, is an exception as it is the only intracellular PG classified to date and the only PG that carries heparin (Iozzo, 1998) (Mulloy, *et al.,* 2017). Proteoglycans can also be divided based upon their localisation: extracellular, including the SLRP decorin and modular PGs aggrecan and perlecan, cell surface-associated, pericellular, and intracellular PGs as seen in *Table 1* (Kolset & Pejler, 2011).

***Table 1: The four families of proteoglycans based upon their classification and known predominant GAGs**; adapted from* (Iozzo & Schaefer, 2015)*. NG2: neuron glial antigen 2, Hep: heparin, HS: heparan sulphate, CS: chondroitin sulphate, KS: keratan sulphate and DS: dermatan sulphate.*

Location	Classification	Eponym	Predominant GAG
Intracellular	Secretory granules	Serglycin	Hep
		Syndecan, 1-4	HS
	Transmembrane	NG2	CS
Cell surface		Betaglycan	HS/CS
		Phosphacan	CS
	GPI-Anchored	Glypican, 1-6	HS
	Basement membrane zone	Perlecan	
Pericellular		Agrin	HS
		Collagen XVIII	
		Collagen XV	CS/HS
		Aggrecan	CS/KS
	Hyalectin/Lectican	Versican	
		Neurocan	CS
		Brevican	
		Biglycan	CS
		Decorin	DS
Extracellular		Fibromodulin	KS
	Canoncial SLRPs	Lumican	KS
		Keratocan	DS/CS
		Osteoadherin	KS
		Epiphycan	DS/CS
	SPOCK	Testican, 1-3	HS

The most abundant family of proteoglycans are extracellular PGs, as seen above in *Table 1,* they are upstream of many major signalling pathways and capable of affecting intracellular phosphorylation events (Mythreye & Blobe, 2010). Extracellular PGs have critical roles in tissue organisation, cell development, growth and maintenance (e.g. epidermal growth factor (EGF) (Bill, *et al.,* 2004) (Kang, *et al.,* 2014), fibroblast growth factor (FGF) (Mundhenke, *et al.,* 2002) (Balasubramanian & Zhang, 2015), hepatocyte growth factor/scatter factor (HG/SF) (Bechard, *et al.,* 2001) (Catlow, *et al.,* 2003), neurotrophins (Schwartz & Domowicz, 2018), transforming growth factor-β (Border, *et al*., 1992) (Chen, *et al*., 2007) , vascular endothelial growth factor (VEGF) (Chiodelli, *et al*., 2011) and Wnts (Saied-Santiago, *et al*., 2017)), coagulation (Ghatak, *et al*., 2015) *(Sobczak, et al., 2018),* inflammation (e.g. interleukins (Borghesi, *et al*., 1999) chemokines (Proudfoot, *et al*., 2017)), cancer (Nikitovic, *et al*., 2018) (Tzanakakis, *et al*., 2019) and infectious disease (e.g. lyme disease *(Leong, et al., 1998),* malaria (Ying, *et al*., 1997), rabies virus (RABV) (Sasaki, *et al.,* 2018), herpes simplex virus (Laquerre, *et al*., 1998) (Pereira, *et al*., 2016) and Human immunodeficiency virus (HIV) (Patel, *et al*., 1993) (Bugatti, *et al*., 2019).

Glycosaminoglycans are structurally and functionally diverse, with CS, DS, KS, HS and HA expressed or co-expressed in many cell types and locations as PGs (Couchman & Pataki, 2012). The most abundant GAG in the body, chondroitin sulphate, is present in a number of locations, it is a major component of the extracellular membrane (ECM) in the central nervous System (CNS), representing approximately 20% of its total volume (Nicholson & Sykova, 1998). Chondroitin sulphate is also present in PGs on the cell surface of membranes in NG2 (nerve/glial antigen 2) (Leoni, *et al*., 2013) and in the extracellular domain of a receptor-type protein tyrosine phosphatase (Milev, *et al*., 1994), pericellularly secreted in type XV collagen with HS found in basement membranes zones of tissues (Li, *et al*., 2000), found extracellularly in the ECM of the CNS as neurocan (Rauch, *et al*., 2001) and as aggrecan co-expressed with Keratan Sulphate (Roughley & Mort, 2014). Heparan sulphate is expressed on the cell membranes in syndecan 1-4 (Szatmari & Dobra, 2013), present in epithelial cells (syndecan 1) (Saunders, *et al*., 1989), mesenchymal cells (syndecan 2) (Marynen, *et al*., 1989), neuronal tissue and cartilage (syndecan 3) (Carey, *et al*., 1992) (Gould, *et al*., 1992) and ubiquitously (syndecan 4) (Vuong, *et al*., 2015) and in glycosylphospatidylinositol-anchored (GPI) proteoglycans, glypicans 1-6 (Condomitti & de Wit, 2018), pericellularly as agrin (Verbeek, *et al*., 1999), type XVIII collagen (Halfter, *et al*., 1998) and perlecan (Yamashita, *et al*., 2018) and extracellularly as testican 1-3 (Schnepp, *et al*., 2005). Keratan sulphate is co-expressed with CS extracellularly as aggrecan (Roughley & Mort, 2014), fibromodulin (Lauder, *et al*., 1997), lumican (Dunlevy, *et al*., 2000) and osteoadherin (Sommarin, *et al*., 1998). Dermatan sulphate is expressed extracellularly as the SLRP decorin (Trowbridge & Gallo, 2002) and co-expressed with CS as epiphycan (Iozzo & Murdoch, 1996). Heparin is only

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expressed in mast cells and basophil granules bound to a serine-rich protein core as the serglycin proteoglycan (Kolset & Pejler, 2011). In mast cells serglycin interacts with carboxypeptidase, chymase, histamine and tryptase, in cytotoxic T cells with granzyme B, endothelial cells with tissue-type plasminogen activator (tPA), macrophages with tumour necrosis factor-α (TNF-α) to ensure their retention inside secretory vesicles and storage granules (Kolset & Tveit, 2008) (Douaiher, *et al*., 2014).

Proteoglycans are integral to a number of physiological processes, such as ligands for small protein growth factors, cytokines, chemokines and morphogens responsible for the regulation of inflammatory responses, cell-cell communication and embryonic development (Iozzo & Schaefer, 2015) (Proudfoot, *et al*., 2017). The characteristics of the proteoglycan that are necessary for this physiological action are mainly conferred by the glycosaminoglycans bound to the protein core (Prydz, 2015).

1.2. Glycosaminoglycans

Glycosaminoglycans are linear and heterogenous glycans, comprised of repeating disaccharide units, alternating between hexosamine and uronic acid, resulting in a structurally complex polysaccharide backbone (Afratis, *et al*., 2012). The biological activity of GAGs depends on a number of properties, including, molecular weight, monosaccharide constituents and the bonds between disaccharide linking units, these are shown below in *Table 2* (Soares da Costa, *et al*., 2017).

Of all the properties associated with GAGs, perhaps the most important is the negative charge (Vallet, *et al*., 2021). Generally, this negative charge is a result of sulphate groups, present on the monosaccharide units or along the GAG backbone, with one exception: hyaluronic acid (Gandhi & Mancera, 2008). The negative charge associated with HA is due to glucuronic acid (Sze, *et al*., 2016), interestingly, HA is the only GAG that does not covalently bind to PGs, but is instead directly secreted into the ECM (Neves, *et al*., 2020). The UA units can be β-D-glucuronic acid or its C5 epimer, α-L-iduronic acid, the latter is a unique hexuronic acid typically found in GAGs whereas D-glucuronic acid is common in nature (Raedts, *et al*., 2011). The epimerisation of D-glucuronic acid is mediated by a D-glucuronyl C5-epimerase acting at a polysaccharide level following glucuronic acid incorporation (Lindahl, *et al*., 1972). The biosynthesis of D-glucuronic acid results in the specific binding properties of glycosaminoglycans: HS and heparin (Shriver, *et al*., 2012) (Lindahl, *et al*., 1979). Heparin is comprised of the repeating disaccharide monomer units of l-iduronate and predominantly N-sulphated d-glucosamine, whereas HS has a structure predominantly comprised of N-sulphated or N-acetylated d-glucuronic acid and d-glucosamine (Shriver, *et al*., 2012). These structural characteristics, epimerisation of uronates, hexosamine units and level of sulphation and acetylation, lead to distinct glycosidic linkage geometry and structure-meaning heparin and HS are fundamentally different (Meneghetti, *et al*., 2015). Hexosamine sugars can be either galactose based: *N*-acetyl-β-D-galactosamine (GalNAc) or glucose based α-D- or β-D-glucosamine (Pomin & Mulloy, 2018).

Table 2: The six glycosaminoglycans and their respective backbone repeat structures, adapted from (DeAngelis, et al., 2013). GlcA: glucuronic acid, IdoA: iduronic acid, GalNAc: N-acetylgalactosamine, GlcNAc: N-acetylglucosamine. $Y=Ac$, SO_3^- or H, $X=$ OH or SO_3^-, with the exception of HA sulpho groups can be found at various O-positions in the sugar ring.
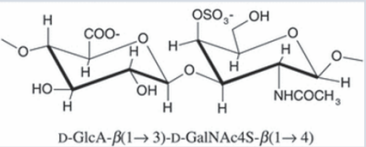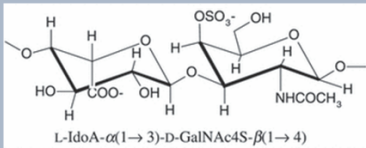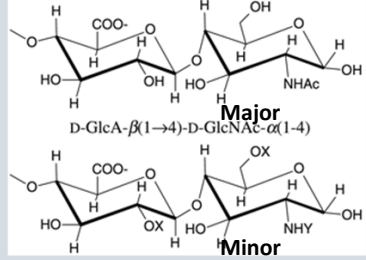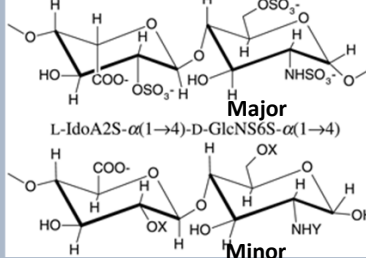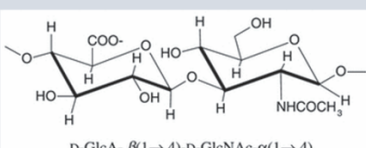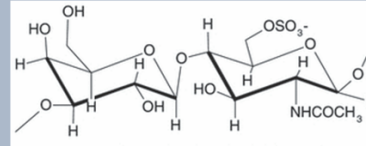
GAG	Major backbone repeat structures
Hyaluronan, hyaluronic acid	$[-4-GlcA\beta-1-3-GlcNAc-\beta1-]_n$
Chondroitin sulphate	$[-4-GlcA2X\beta-1-3-GalNAc4X6X-\beta1-]_n$
Dermatan sulphate	$[-4-IdoA\alpha$ **or** $GlcA2X$ $\beta-1-3-$ $GalNAc4X6X-\beta1-]_n$
Heparan sulphate	$[-4-GlcA\beta$ or $IdoA2X\alpha-1-4-$ $GlcNY3X6X-\alpha1-]_n$
Heparin	$[-4-IdoA\alpha$ or $GlcA2X\beta/-1-4-$ $GlcNY3X6X-\alpha1-]_n$
	$[-3Gal\beta1-4GlcNAc\beta1]n$
Keratan sulphate	$\beta-1-4-Gal-\beta-1-3-GlcNAc$

The permutation of these monosaccharide units within the GAG backbones results in the different GAG families, the GlcN-containing heparin and heparan sulphate (Sasiekharan & Venkataraman, 2000) (Rabenstein, 2002) and the GalNAc-containing chondroitin sulphate and dermatan sulphate. Keratan sulphate alternates *N*-acetyl-glucosamine with Galactose (Gal) and does not contain any UA units (Funderburgh, 2000) (Pomin, 2015), hyaluronic acid (HA) or hyaluronan, alternates GlcNAc with GlcA and lacks sulphation and a protein core (Almond, 2007). The major backbone repeats of these structures are visualised below in *Table 3*.

Cell and tissue specific GAGs are produced by covalent modification by incomplete enzymatic modifications to the glycan backbone (Soares da Costa, *et al.*, 2017). Chondroitin sulphate is comprised of repeating GalNAc-GlcA disaccharide units, joined by β1,4 and β1,3 linkages respectively, CS can be *O*-sulphated at the C4 (CS-A), C6 (CS-C) or both positions of the GalNAc unit (CS-E), as seen in *Table 2* (Mikami & Kitagawa, 2013). The GlcA unit can also be sulphated at the C6 position resulting in 6-sulphated GalNAc and 2-sulphated GlcA (CS-D) (Mikami & Kitagawa, 2013). Dermatan sulphates are stereoisoMERS-CoV of chondroitin sulphates, a result of enzymatically driven C5 inversion of differing amounts of GlcA to IdoA (Soares da Costa, *et al.*, 2017). Dermatan sulphate consists of GalNAc-IdoA units joined by β1,4 and α1,3 linkages respectively (Silbert & Sugumaran, 2002). The degree of sulphation of DS varies between one and three per disaccharide unit, the sulphation is catalysed by the same enzymes as in CS biosynthesis (Lindahl & Hook, 1978). There is only one specific DS sulphation enzyme, that catalyses *O*-

sulphation at the C4 position of GalNAc (Funderburgh, 2008), this occurs immediately after C5 epimerisation, preventing the reversible epimerisation of IdoA (Malmstrom, 1981). Similar to CS, many of the DS IdoA units undergo *O*-sulphation at the C2 position of IdoA, the sulfotransferase responsible for this has higher activity toward IdoA units as in DS than GlcA in CS, as a result 2-*O*-sulphation is more abundant in CSs (Soares da Costa, *et al*., 2017). Keratan sulphate consists of repeating Gal and GlcNAc joined by by β1,4 and β1,3 linkages respectively, there are 3 forms of KS, distinguished by their difference in oligosaccharide to protein core linkages (Funderburgh, 2008). Sulphation patterns of KS vary in degree, GlcNAc can be 6-*O*-sulphated, this action is essential for the elongation of the KS chain, with sulphation and elongation occurring simultaneously, whereas Gal can be sulphated following chain elongation at the C6 position (Fukuta, *et al*., 1997). Hyaluronic acid is unique in that it is neither sulphated nor covalently bound to a protein core, instead it interacts with PGs through a HA binding protein (Bignami, *et al*., 1993) (Laurent & Fraser, 1992).

***Table 3: The repeating disaccharide units of the six various glycosaminoglycans**: including the major and minor structures of heparan sulphate and heparin, defining structural features and physiological location. (X=H or SO_3^-, Y=Acetylation, SO_3^- or H). Adapted from (Gandhi & Mancera, 2008)*

Glycosaminoglycan	Disaccharide units	Features
Chondroitin sulphate	 D-GlcA-β(1→ 3)-D-GalNAc4S-β(1→ 4)	Mw: 5-50 kDa Most abundant GAG in the body, found in cartilage, tendons, ligaments, cornea, and aorta Binds to proteins, like collagen to form proteoglycan aggregates
Dermatan sulphate	 L-IdoA-α(1→ 3)-D-GalNAc4S-β(1→ 4)	Mw: 15-40 kDa Found in the cornea and sclera of the eye, blood vessel walls, heart valve, and the umbilical cord Binds to proteins, like collagen to form proteoglycan aggregates to form the PG decorin
Heparan sulphate	 **Major** D-GlcA-β(1→4)-D-GlcNAc-α(1-4) **Minor**	Mw: 10-70 kDa Extracellular componemt found in the basement membrane and a ubiquitous component of cell surfaces Sulphate vs hexosamine content: 0.8-1.8 α-L-iduronic acid content: 30-50%
Heparin	 **Major** L-IdoA2S-α(1→4)-D-GlcNS6S-α(1→4) **Minor**	Mw: 10-12 kDa Intracellular component of mast cells particularly in the liver, lungs and skin Sulphate vs hexosamine content: 1.8-2.4 α-L-iduronic acid content: >70%
Hyaluronic acid	 D-GlcA- β(1→ 4)-D-GlcNAc-α(1→ 4)	Mw: 4-8000Da Non-sulphated, non-covalently bound to proteins in the ECM Typically found in the synovial fluid, articular cartilage, vitreous humour and loose connective tissue. Present in the capsules of certain microbial strains (e.g., strains of streptococci)
Keratan sulphates I and II	 D-Gal-β(1→ 4)-D-GalNAc6S-β(1→ 3)	Mw: 4-19 kDa Most heterogenous GAG KS I is found in the cornea KS II is found in the cartilage aggregated with CS Another rich source is the brain tissue

1.3. Heparin and heparan sulphate:

1.3.1. Structure

Disaccharides of HS and heparin are composed of alternating α1,4 GlcNAc and 4-linked β1,4 GlcA units, as seen in *Figure 1* (Sasiekharan & Venkataraman, 2000) (Rabenstein, 2002), structurally, they differ only in the relative proportions of these monosaccharide and disaccharide structures (Pomin & Mulloy, 2018). Constituent disaccharides undergo a series of extensive enzymatic modifications that are concomitant and independent, however, this process does not appear to be directly template driven (Linhardt, *et al*., 2007). This results in highly heterogenous glycans, variable in chain length, size, space and extent of epimerisation and sulphation (Meneghetti, *et al*., 2015). Modifications of HS, unlike CS, occur in clusters instead of in long regions, this results in segments of sulphation separating unmodified regions, which gives HS and heparin their specific binding properties (Esko & Selleck, 2002) (Capila & Linhardt, 2002).
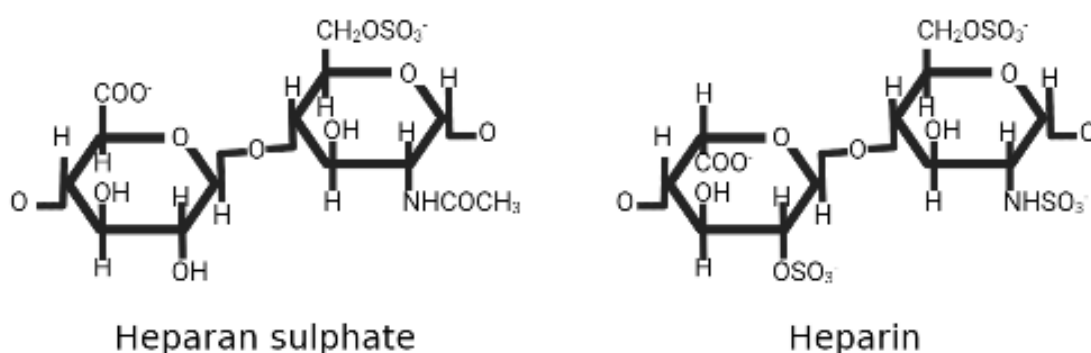
Figure 1: Structure of the predominant heparin and heparan sulphate disaccharides, created using (Biorender.com, 2022)

In heparin the D-glucosamine units are predominantly *N*-sulphated, whereas in HS they are *N*-acetylated. Heparin is mainly composed (~ 70-80%) of 2-sulfated IdoA units (IdoA2S) together with *N*,6-di-sulphated GlcN units (GlcNS6S) (*N*-sulfoglucosamine/2-*O*-sulphated IdoA (-GlcNS6S-IdoA2S-)). More rarely, 3-*O*-sulphation at the GlcNS6S units can also occur (GlcNS3S6S) in both HS and heparin but this is more common within heparin chains, this sulphation results in a high affinity for antithrombin and high anticoagulant activity in Heparin (Capila & Linhardt, 2002) (Mulloy, *et al*., 2016). Whereas in HS, the predominant disaccharide unit is glucuronic acid/glucosamine (~40-60%) which can be either *N*-acetylated or *N*-sulphated (Turnbull, 2002). However, lower amounts of *N*-sulphated glucosamine (GlcNS) and rare amounts of unsubstituted GlcN can also occur- this appears to be an artefact of scientific investigation rather than a natural modification (Pomin & Mulloy, 2018). HS has a higher average molecular weight (50kDa) than heparin (20kDa) (Mulloy, *et al*., 2000), a lower degree of epimerisation and sulphation more negatively charged, depending on chain length (Gallagher & Walker, 1985) (Casu, 1989).

Heparin is a linear glycopolymer, consisting of pyranose rings that can adopt a variety of structures depending on ring substitutions and adjacent ring modifications, often in energetically favourable conformations (Ma & Zhu, 2019). Conformational studies show that α1,4 GlcNAc and β1,4 GlcA are in the regular $^4C_1$ conformation (Pomin, 2011). In this conformation, GlcN units have the glycosidic bond in the axial conformation (perpendicular to the plane of the pyranose ring) and the aglycone bond (a noncarbohydrate group combined with a sugar to form a glycoside) in equatorial orientation (in the plane of the pyranose ring) (Marszalek, *et al*., 2003). Whereas, in β-D-GlcA units, both bonds are in the equatorial orientation (Marszalek, *et al*., 2003). IdoA is more flexible and can adopt both chair $^1C_4$ and skew boat $^2S_0$ conformations, more commonly the former (Hsieh, *et al*., 2014) (Sattelle & Almond, 2011). These conformations are visualised in *Figure 2*. This is essential for the binding of HS to antithrombin (Kas, *et al*., 2001), this provides the anticoagulant activity and ability to regulate cell growth by fibroblast growth factors among other notable physiological functions (Raman, *et al*., 2003).
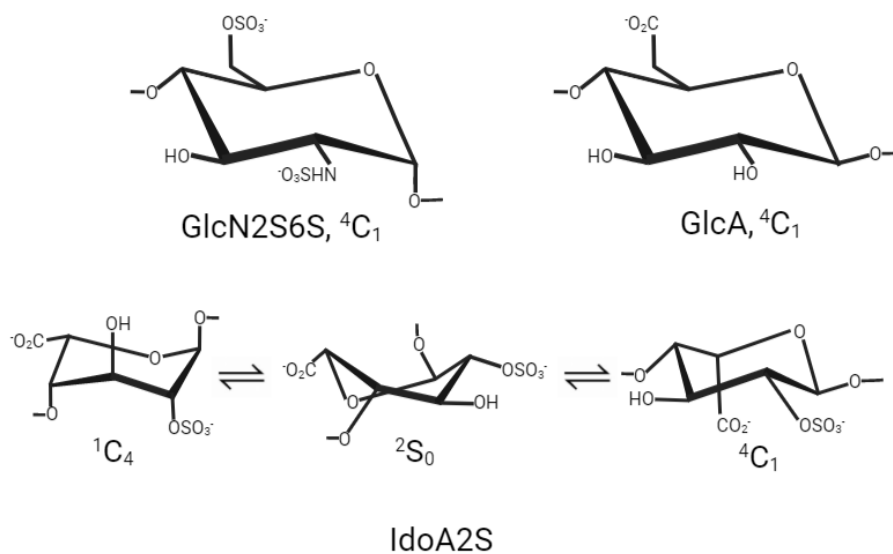
Figure 2: Different conformations of the possible ring structures present in heparin. A: GlcN2S6S and GlcA and B: the conformation equilibrium of IdoA2S observed in heparin, adapted from (Meneghetti, et al., 2015).

Heparin is sometimes thought of as a more highly sulphated and specialised HS, as a result of these characteristics it has less structural variability (Shriver, *et al*., 2012). Despite this, there are areas of lower sulphation spread throughout the heparin chain (Yates, *et al*., 2019). Due to differential sulphation and variation in chain length, the structure of HS is highly variable (Meneghetti, *et al*., 2015). This is a result of the non-template driven manner of biosynthesis, through enzymatic modifications by C5-epimerase, O-sulfo-transferases and N-deactylase/N-sulfotransferase (Salmivirta, *et al*., 1996) (Turnbull, *et al*., 2001). The heterogeneity of HS is dependent on cell type, tissue type and/or the degree of cell differentiation, this accounts for the high level of variation in biological functions with integral roles in the regulation of cellular signalling (Nagamine, *et al*., 2012). The variability of structure within heparin, although low, is important as it provides heparin with bioactivity for which it is primarily known, that of anticoagulant action (Oduah, *et al*., 2016). This activity is a result of the potentiating action on an anticoagulation factor, antithrombin (ATIII) (Lu, *et al*., 2017). Antithrombin non-covalently binds to pro-coagulation factors, thrombin and Xa inhibiting their actions (Dydek & Chaikof, 2016). The anticoagulant activity of heparin is a result of motifs within the structure of heparin that bind with high affinity to ATIII, whilst allowing the inhibition of factor Xa and thrombin (Desai, 2004). The motifs responsible for this affinity have not been fully elucidated yet, but the most studied is a pentasaccharide sequence (Thacker, *et al*., 2013). This motif contains three glucosamine residues and two uronic acids, with a 6-O-sulphation on the non-reducing end glucosamine, the glucuronic residue, N- and 3-O-sulphation on the central glucosamine, followed by the iduronic acid and the N-sulphate on the glucosamine reducing end,

as visualised below in *Figure 3* (Sankakaranarayanan, *et al*., 2020). Only ~one-third of all heparin chains contain this 3-O-sulphation event, that augments heparin-antithrombin binding (Nutsecu, *et al*., 2016).
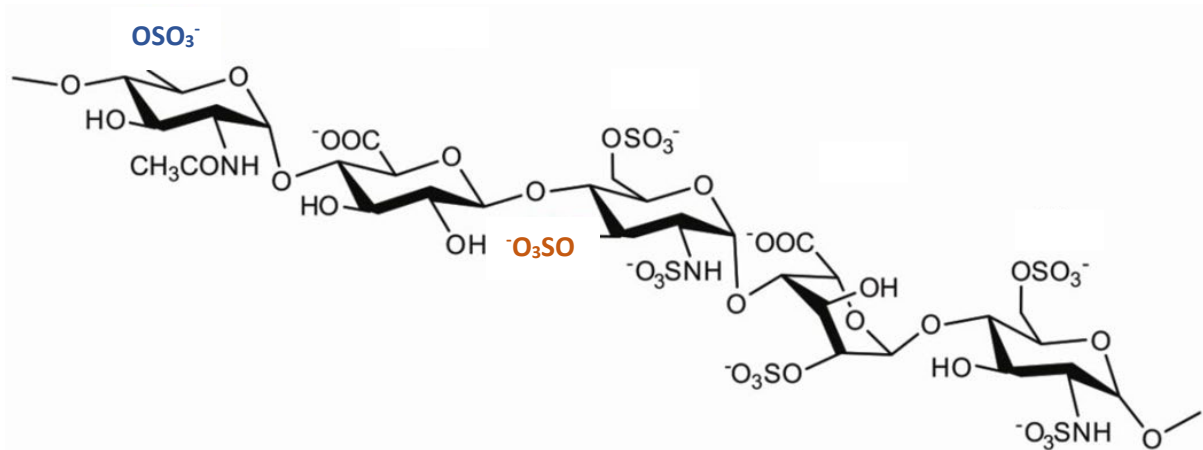
Figure 3: Structure of the antithrombin binding pentasaccharide sequence found in heparin. *The central 3-O-sulphate group generated by Hs3st-1 (shown in orange) and the 6-O-sulphate group at residue −2 (shown in blue) account for the majority of the binding energy of antithrombin to heparin, adapted from (Thacker, et al., 2013).*

1.3.2. Biosynthesis:

Biosynthesis of heparin and HS occurs in the endoplasmic reticulum and the Golgi, with the process consisting of three broad phases: initiation, polymerisation and modification of the chain (Chandarajoti, *et al*., 2016). Unlike proteins and nucleic acids, the biosynthesis of heparin and heparan sulphate does not appear to be directly template driven (Meneghetti, *et al*., 2020). Despite many research attempts, the biosynthetic route, the activity of biosynthetic enzymes, their substrate specificities, has not yet been fully elucidated (Kreuger & Kjellen, 2012) (Rudd & Yates, 2012). Currently a viable biosynthetic pathway that is able to account for the structural heterogeneity of heparin and heparan sulphate whilst being consistent with experimental observations is widely accepted, as seen below in *Figure 4* (Meneghetti, *et al*., 2020).

The biosynthesis of heparin is initiated by the linking of a tetrasaccharide linkage saccharide comprised of two galactose, glucuronic acid and xylose: glucuronic acid-galactose-galactose-xylose [GlcUAβ1-4GlcNAcα1-]$_n$ at a serine residue of the proteoglycan core serglycin (Carlsson, *et al*., 2008). Following this, chain polymerisation occurs by the sequential addition of D-glucuronic acid (1-4) *N*-acetyl-D-glucosamine disaccharide units, catalysed by exostosin glycosyltransferase 1 (EXT1) and EXT2 polymerases (Lind, *et al*., 1998). The newly generated [4GlcUAβ1-4GlcNAcα1-]$_n$ polysaccharide chain is subsequently modified by partial N-deacetylation and N-sulphation along the chain of select GlcNAc units by a family of dual functioning N-deacetylase/N-sulfotransferases (NDSTs) *(Salmivirta, et al., 1996)*. This enzyme converts GlcNAc to GlcNS, this NS region is required for further modifications, including epimerisation and O-sulphation (Deligny, *et al*., 2016). *N*-sulphated glucosamine units are found in contiguous stretches, interspersed by non-modified *N*-acetylated regions (Maccaran, *et al*., 1996). Regions of alternating GlcNS and GlcNAc units (NA/NS) surround the NS domains (Esko & Lindahl, 2001). The next step is widely agreed to be the catalysation of GlcA to IdoA by a C5-epimerase, this is specific to GlcA residues at the reducing end of GlcNS in the glycan chain as this is vital for substrate recognition of GlcA by C5-epimerase (Hagner-McWhirter, *et al*., 2004). 2-O-sulfotransferase catalyses the transfer of an O-sulphate group to the 2-*O*-position of either IdoA or GlcA, but preferentially to the IdoA residue (Rudd & Yates, 2012). GlcNS can be modified by 6-*O*-sulfotransferase by one of three 6OSTs, however, the presence of IdoA(2S)-GlcNS(6S) is abundant in heparin, this indicates 6-*O*-sulphation follows 2-*O*-sulphation (Rabenstein, 2002). More rarely, modifications requiring 2-*O*-sulphation of GlcA, 6-*O*-sulphation can occur in the GlcNAc adjacent to a disaccharide comprised of a GlcNS and GlcNS(6S) can be 3-*O*-sulphated this is critical for AT binding and high anticoagulant activity (Wang, *et al*., 2017) (Yates, *et al*., 2019).
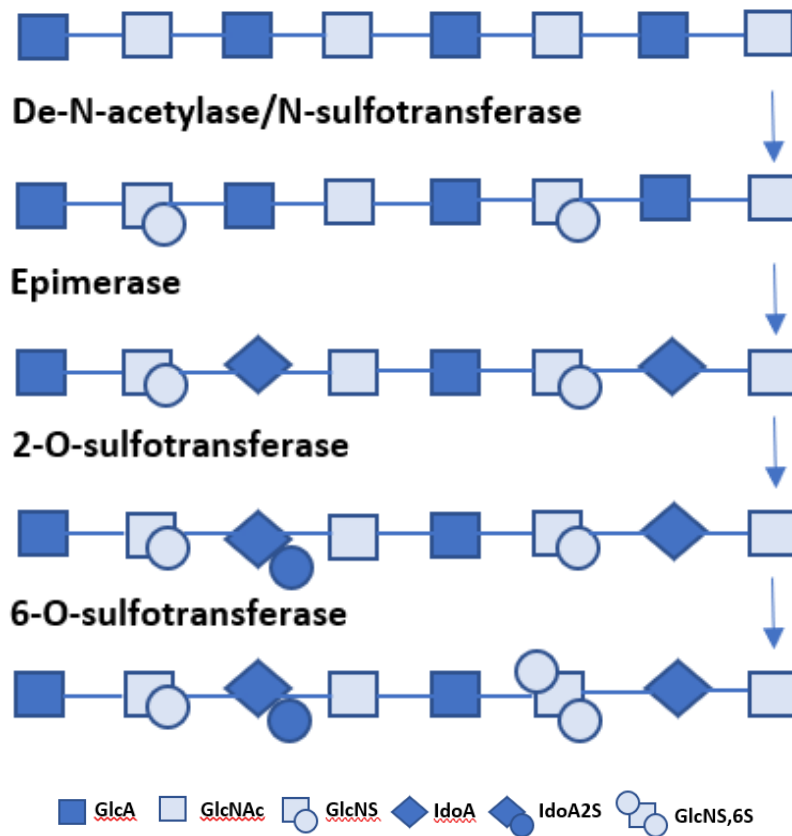
Figure 4: The conventional depiction of the heparan sulphate/heparin biosynthetic pathway, adapted from (Rudd & Yates, 2012).

Heparan sulphate formation occurs very similarly to heparin, however, it is less sulphated (Meneghetti, *et al*., 2015). Heparan sulphate contains all of the structural variations as in heparin but the frequency at which these minor variations occur is much greater, accounting for HSs structural heterogeneity (Shriver, *et al*., 2012). Biosynthesis occurs in a similar manner, with the generation of a nascent [4GlcUAβ1-4GlcNAcα1-]$_n$ polysaccharide chain, which is first subjected to partial N-deacetylation/N-sulphation of GlcNAc residues (Safaiyan, *et al*., 2000). Modification to the chain occurs in a regioselective manner, creating three distinct regions: NS domains (consecutively N-sulphated regions), NA/NS domains (regions of alternating N-acetylated and N-sulphated disaccharide units) and NA domains (unmodified regions, remaining N-acetylated) (Safaiyan, *et al*., 2000).

2. Uses of heparin as inhibitors or potential treatments of viral infection

2.1. Biological Activity of heparan sulphate and heparin

Heparan sulphate and heparin are involved in a plethora of extracellular signalling events influencing, cell and tissue development (Linhardt & Toida, 2004) (Bishop, *et al*., 2007). It has been found that that the HS sites containing stretches of GlcNS and a high proportion of IdoA are involved in protein binding (Powell, *et al*., 2004). In particular, a higher GlcNS and IdoA content appears to be favourable in fibroblast growth factors (FGFs) and fibroblast growth factor receptors (FGFRs), however, this may be an artefact of experimental preparation and isolation of oligosaccharides (Powell, *et al*., 2004). The conformational flexibility of Ido2AS residues is assumed to be essential for the binding of biologically important proteins such as antithrombin (Das, *et al*., 2001) and regulation of cell growth by FGFs (Raman, *et al*., 2003). Heparin and HS interact with these heparin binding proteins by hydrogen or ionic bonds between sulphate groups of heparin and HS and amino groups of proteins (Bolten, *et al*., 2018). Iduronic acid residues can exist in both $^1C_4$ chair and $^2S_0$ conformation, as seen in *Figure 2*, this allows for appropriate electrostatic interactions with basic amino acids of proteins that bind to heparin and HS (Ferro, *et al*., 1990) (Gallagher, 2006) (Ragazzi, *et al*., 1993).

The ionic interactions between the carboxyl and sulphate groups of GAGs and basic amino acids (e.g., lysine and arginine) residues in proteins, are the main contributors to the formation of HS and heparan-protein complexes (Cardin & Weintraub, 1989). However, in some cases there are significant contributions by non-ionic interactions, through hydrogen bonding (Singh, *et al*., 2011), hydrophobic forces (Zhao, *et al*., 2018) and van der Waals forces (Raman, *et al*., 2003). Binding results in protection of proteins from degradation (Sommer & Rifkin, 1989), conformational change of the protein to activate or deactivate its action (e.g. in antithrombin) (Desai, *et al*., 1998) and clustering of binding complexes at the cell surface (Torrent, *et al*., 2012). In all cases, the resulting heparin/HS-protein complexes are integral to processes such as cell growth and migration, affecting the development of tissues and organs (Soares da Costa, *et al*., 2017).

Heparin interacts with over 250 proteins physiologically in homo sapiens (peer reviewed results included only) (UniProt, 2021), these include, fibroblast growth factors, amyloid-like protein 1 and proteins involved in coagulation such as antithrombin-III, coagulation factor XI and platelet factor-4 (UniProt, 2021). Bioinformatic research carried out by, (Ori, *et al*., 2011) revealed that heparan sulphate interacts with 435 human proteins, some of which are displayed below in *Table 4*. The extent of the heparan sulphate interactome is a result of their distribution in various tissues, heparan

sulphate is ubiquitously found at the cell surface and extracellular matrix of all animal species, whereas heparin is found exclusively inside storage vesicles of mast cells (Dreyfuss, *et al*., 2009).

Table 4: Heparan sulphate binding proteins, adapted from (Dreyfuss, et al., 2009).

Cell surface	References
FGF receptor	(Schultz, et al., 2017)
HIP (Heparin/Heparan Sulphate Interaction Protein)	(Rohde, et al., 1998)
L-selectin and P-selectin	(McEver & Cummings, 1997)
MAC-1 (Monocyte Adhesion Molecule)	(Zen, et al., 2009)
N-CAM (Neural Cell Adhesion molecule)	(Storm, et al., 1996)
PECAM-1 (Platelet endothelial Cell Adhesion Molecule)	(dela Paz, et al., 2014)
Cytokines/Chemokines/Morphogens	
BMP (Bone morphogenetic protein)	(Kuo, et al., 2010)
CCL-2 (CC-chemokine ligand)	(Crijns, et al., 2020)
GM-CSF (Granylocyte Macrophage Colony Stimulating Factor)	(Sebollela, et al., 2005)
Hh (Sonic Hedgehog)	(Chang, et al., 2011)
IL-1, -2, -3, -4, -5, -7, -8, -10, -12 (interleukin)	(Salek-Ardakani, et al., 2000)
IP-10 (Interferon-γ inducible protein 10)	(Soejima & Rollins, 2001)
MCP-1, MCP-4 (Monocyte Chemoattractant Protein)	(Deshmane, et al., 2009)
MIP-1 (Macrophage Inflammatory Protein)	(Stringer, et al., 2003)
PF-4 (Platelet factor 4)	(Fiore & Kakkar, 2003)
RANTES (Regulated on Activation Normal T cell Expressed and Secreted)	(Burns, et al., 1999)
TNf-α (Tumour Necrosis Factor)	(Bode, et al., 2005)
Wnt (Wingless wg)	(Mii, et al., 2017)
Extracellular Matrix	
Collagens	(Sweeney, et al., 1998)
Fibronectin	(Raitman, et al., 2017)
HB-GAM (heparin Binding Growth Associated Molecule)	(Kinnunen, et al., 2008)
Laminin	(Schuger, et al., 1996)
Tenascin	(Midwood & Orend, 2009)
Thrombospondin I and II	(Feitsma, et al., 2000)
Vitronectin	(Wilkins-Port & McKeown-Longo, 1996)
Growth factors	
FGF family (Fibroblast Growth Factors)	(Galanternik, et al., 2015)
HB-EGF (Heparin Binding- Epidermal Growth Factors)	(Dao, et al., 2018)
HDGF (Hepatoma Derived Growth Factor)	(Wang, et al., 2010)
HGF (Hepatocyte Growth Factor)	(Hartmann, et al., 1998)
PDGF (Platelet-Derived Growth Factor)	(Abramsson, et al., 2007)
PIGF (Placenta Growth Factor)	(Athanassiades & Lala, 1998)
TGF-β (Transforming Growth Factor β)	(Rider, 2006)
VEGF (Vascular Endothelial Growth Factor)	(Stringer, 2006)
Others	
Angiogenin	(Soncin, et al., 1997)
Annexin V	(Capila, et al., 2001)
Cathepsins B and G	(Almeida, et al., 2001)
DNA and RNA polymerases	(Chammas, et al., 2017)
Myosin ATPase	(Tersariol, et al., 1992)
Na+/Ca2+ exchanger protein	(Shinjo, et al., 2002)
Neutrophil elastase	(Xu, et al., 2015)
Prion	(Imamura, et al., 2016)
Superoxide dismutase	(Adachi, et al., 2001)
β-amyloid protein	(Shimizu, et al., 2009)

Heparan sulphate proteoglycans (HSPGs) have a variety of functions, a result of their structural differences due to their varied cell and tissue type and location. HSPGs are found at the cell surface in the ECM, here they interact with a wide variety of ligands, acting as coreceptors, facilitating the formation and signalling of FGF2-FGF receptor complexes (Rapraeger & Olwin, 1991), supported by further research, showing that knockout of NDST-1 decreases binding of FGF-2 and $VEGF_{164}$ to HS (Fuster, *et al*., 2007). HSPGs are known to act as endocytic receptors (Belting, 2003), syndecan HSPGs have been found to mediate internalisation of insulin regulated aminopeptidase (IRAP) (Fuki, *et al*., 2000), they have also known to bind to guanidinylated neomycin (GNeo), to transport high molecular weight into the lysosomal compartments of cells (Sarrazin, *et al*., 2010). Syndecans 2, and 4 have been shown to act as adhesion receptors, binding a number of ligands through their HS chains (Oh & Couchman, 2004), supported by research showing that when in fibroblasts lacking syndecan-4, decreasing the number and size of focal adhesions (Gopal, *et al*., 2010). Roles in regulating growth factor binding to ECM and cell migration, HSPGs, glypican-4 and syndecan-4 are integral to cell movement during gastrulation and migration of neural crest cells respectively by binding platelet-derived growth factor (PDGF) (Smith, *et al*., 2009). Similarly HS-dependent interactions between vascular endothelial growth factor (VEGF) have been reported, with sulphation of the 6-*O* and *N* positions of glucosamine required for full activity (Mitsi, *et al*., 2006) and morphogen and chemokine gradients, it has been found that glypicans are vital for morphogen diffusion (Han, *et al*., 2005) (Yan & Lin, 2009).

Annexins, a family of homologous proteins are widely distributed and ubiquitous in eukaryotes (Benz & Hofmann, 1997) have been implicated in a number of functions including cell signalling, membrane trafficking, inflammation and blood coagulation (Seaton & Dedman, 1998) (Gerke & Moss, 1997). Calcium-dependent GAG binding to annexins has been widely studied and characterised (Kassam, *et al*., 1997). Annexin V is known to bind a heparin-derived tetrasaccharide in the $^2S_0$ conformation, whilst the non-interacting tetrasaccharide is in the $^1C_4$ conformation (Capila & Linhardt, 2002) (Ishitsuka, *et al*., 1998). This demonstrates the importance of the Ido2AS flexibility in conferring specific binding properties of heparin and HS. The conformation of IdoA varies, dependent on the substitution pattern of the residue and its position in the chain (Sanderson, *et al*., 1987). When at the reducing end, Ido2AS has three possible conformations, $^4C_1$. $^1C_4$ chair and the $^2S_0$ skew boat (Sanderson, *et al*., 1987), internal Ido2AS exists in an equilibrium between the chair and skew boat (Mikhailov, *et al*., 1997). Of these two conformations, the skew boat, $^2S_0$, is favoured, being more stable and minimises unfavourable 1,3 diaxal unbonded interactions present

in the chair form (Mikhailov, *et al*., 1996). However, to change to the chair form is not impossible as the energy barrier is low, this allows for movement between the two conformers to make the most favourable electrostatic interactions in the protein bound state (Capila & Linhardt, 2002).

One of the largest areas of research with regards to heparan sulphate activity is the involvement of heparan sulphate in fibroblast growth factor signalling (Harmer, 2006). This process begins with the formation of a ternary complex of FGF, FGFRs with intracellular tyrosine kinase domains and heparan sulphate (Ornitz & Itoh, 2015). The signalling involves ligand-induced receptor dimerisation and autophosphorylation, followed by a downstream transfer of the signal (Lemmon & Schlessinger, 2010). The binding of heparan sulphate, as a HSPG or heparin to FGFs and FGFRs enhances FGF signalling by mediating the complex formation between FGFs and FGFRs (Wu, *et al*., 2003). There are 22 known FGFs and five types of FGFRS in humans, with a variety of roles, some of which are important for the modulation of many fundamental cellular processes (Xu, *et al*., 2012). Secreted FGFs are expressed almost ubiquitously, with essential roles in early embryonic development in organogenesis (Kato & Sekine, 1999). In adults FGFs are important for homeostatic factors, with roles in tissue repair, maintenance and regeneration and metabolism and the canonical FGFs control cell differentiation, migration, and proliferation (Nunes, *et al*., 2016). Research aims to identify potent mimetics that can regulate abnormal FGF signalling in malignant states, such as in cancers, by inhibiting the angiogenesis that supports the growth of solid tumours (Ling, *et al*., 2015).

Mast cells (MCs) are key effectors in various inflammatory reactions, most notably allergic reactions (Amin, 2012) but have been implicated in other pathophysiological conditions such as autoimmune diseases including rheumatoid arthritis (Rivellese, *et al*., 2019), tumour metastasis (Aponte-Lopez, *et al*., 2018) and wound healing (Ng, 2010). Studies have shown that upon mast cell activation, the contents of their secretory granules are released, including potent inflammatory mediators: histamine, heparin proteoglycans, cytokine, and many heparin binding proteases such as, carboxypeptidase A, chymases and tryptases (Borish & Joseph, 1992). It has been shown in previous studies, that inactivation of NDST-2 affected MCs severely, altering morphology and complete absence of heparin binding MC proteases at the protein level (Henningsson, *et al*., 2002). It has been shown that inactivation of NDST-2, therefore, the heparin/mast cell protease system, may affect regulation of the coagulation at extravascular sites (Tchougounova & Peljer, 2001).

2.2. Heparin as a potential therapeutic

Although used widely as an anticoagulant, research has shown the potential uses of heparin as a treatment in many pathologies (Hirsh, *et al*., 2001). Protective roles in the inflammation response have been suggested, involving interaction with several cytokines, inhibition of elastase and heparinase resulting in decreased leukocyte recruitment (Page, 2013). As a result, heparin inhibits leukocyte-endothelial adhesion (Bazzoni, *et al*., 1993) (Silvestro, *et al*., 1994) and limits accumulation of cells in inflamed tissues, in both allergic (Seeds & Page, 2001) (Vancheri, *et al*., 2001) and non-allergic inflammation responses (Johnson, *et al*., 2004) (Lever, *et al*., 2010). It has been shown that heparin confers benefits in the treatment of cancer in addition to the direct effects of heparin on coagulation (Borsig, 2010). Trials involving cancer patients undergoing heparin treatment for prophylaxis of venous thromboembolism (VTE) show improved rates of survival (Hettiarachchi, *et al*., 1999). The role of heparin in treating cancer is multifaceted, with effects including but not limited to, the production of thrombin by heparin results in fibrin formation impeding natural killer cell (NK) activity (Ponert, *et al*., 2018), heparin reduces angiogenesis by inhibition of VEGF, tissue factor and platelet activating factor (Battinelli, *et al*., 2014). Heparin also decreases the activation and limits the effects of matrix metalloproteinases (MMPs), serine proteases and heparanases, which have integral roles in metastasis (Coussens & Werb, 2010). Following early reports of *in vitro* inhibition of Herpes simplex virus (Nahmias & Kibrick, 1964), the potential of heparin and sulphated polysaccharides to inhibit HIV was investigated (Rider, 1997). The potential use of heparin as a therapeutic aid to existing cocktails was promising as early *in vitro* data demonstrated that heparin competes with host cell HS surface receptors blocking viral entry (Cagno, *et al*., 2019), however, anticoagulant activity limits dosage (Cassinelli & Naggi, 2016). Similarly, poor pharmacokinetics and oral absorption of heparin were also drawbacks to further development, as the benefit to therapy was outweighed by heparins limited pharmaceutical efficacy in HIV (Cassinelli & Naggi, 2016), despite this low weight molecular heparin (LMWH) has been suggested as a safer more feasible treatment (Howell, *et al*., 1996). Heparin has been found to have inhibitory activity in a number of RNA and DNA viruses *in vitro*, including hepatitis B and C (Zahn & Allain, 2005), Coxsackie virus (Zautner, *et al*., 2006), Varicella-Zoster virus, responsible for chicken pox and shingles (Gershon & Gershon, 2013), influenza (Skidmore, *et al*., 2015), Zika Virus (Ghezzi, *et al*., 2017), Yellow Fever (Germi, *et al*., 2002) and Dengue virus (Lin, *et al*., 2002). Similar obstacles remain for the use of heparin as a treatment in these cases as seen

with AIDs, to combat this research is now being targeted towards the administration of nebulised heparin, with the hopes that the risk of bleeding can be reduced if not eradicated (Haren *et al.,* 2022).

2.3. Anti-inflammatory properties of heparin

Though widely used for its anticoagulant properties, heparin has been known to display several properties that may have clinical benefits. In light of the novel coronavirus pandemic, the anti-inflammatory action of heparin has become more widely researched, as opposed to solely focussing on the anticoagulant properties (Hippensteel, *et al*., 2020) (Shi, *et al*., 2020). Due to the physiological location of heparin, in mast cells, it was thought that endogenous heparin may have a role in the regulation of inflammatory responses (Lever, *et al*., 2016). Physiologically mast cells are known to modulate angiogenesis, innate and adaptive immune responses, vasodilation, and vascular homeostasis (Krystel-Whittemore, *et al*., 2016). Mast cells have been implicated in the pathophysiology of many diseases, including cardiovascular diseases, gastrointestinal disorders, allergies, asthma, all of which are associated with inflammation (Bot, *et al*., 2008) (Ramsay, *et al*., 2010) (Amin, 2012) (Mendez-Enriquez & Hallgren, 2019).

Experimental and clinical studies have illuminated the role of heparin as a potential anti-inflammatory, demonstrating the broad inhibitory activity of heparin at multiple stages of inflammatory responses. (Bendstrup & Jensen, 2000) (Thourani, *et al*., 2000). Heparin has inhibitory activity against inflammatory cell recruitment including neutrophil activation and some research has demonstrated an inhibitory role in mast cell degranulation (Brown, *et al*., 2003), the process leading to transendothelial migration of neutrophils- rolling and adhesion (Riffo-Vasquez, *et al*., 2016), platelet leucocyte interactions (Lappegard, *et al*., 2004) and inhibition of heparanase (HSPE) activity (Cassinelli, *et al*., 2020). Heparin has also been shown to inhibit inflammation mediators which propagate the inflammatory response subsequently causing tissue damage and remodelling (Spencer, *et al*., 2006) (Ogawa, *et al*., 2013).

The potential of heparin for use in this way has not been exploited due to the potential adverse effects, these include heparin-induced thrombocytopenia (HIT) and heparin-induced thrombocytopenia with thrombosis (HITT), heparin-associated osteoporosis and haemorrhagic complications (Franchini, 2005)(Wawrzynska, *et al*., 2003). Heparin-associated osteoporosis is rare and associated with long-term therapy, often occurring during pregnancy or the postpartum period (Wawrzynska, *et al*., 2003). Although haemorrhagic complications are concerning, there is

evidence that the anti-inflammatory activity of heparin is independent of its anticoagulant activity (Lever, *et al*., 2010). Heparin-induced thrombocytopenia and heparin-induced thrombocytopenia thrombosis (HIT/T) are some of the most common adverse effects of heparin treatment (Franchini, 2005). Both of which are caused by the emergence of antibodies that activate platelets in the presence of heparin (Ahmed, *et al*., 2007). Despite thrombocytopenia, bleeding is rare, HIT is more strongly associated with thromboembolic complications in both the arterial and venous systems (Sinan, 2015).

The association of thrombocytopenia and anticoagulation with thromboembolic events can be attributed to disseminated intravascular coagulation (DIC), which is characterised by activation of coagulation pathways to result in intravascular thrombi formation, depletion of platelets and coagulation factors (Levi, 2010). Disseminated intravascular coagulation can be triggered by cell injury or death that subsequently initiates coagulation *in vivo* to produce thromboplastic agents directly, by release of thromboplastic contents or indirectly when monocytes and or endothelial cells are stimulated to produce and secrete cytokines in response to injury, activating the extrinsic pathway of coagulation (Kitchens, 2009). Heparin induced thrombocytopenia should always be considered as a differential diagnosis for DIC as the presentations can be similar, thrombocytopenia of DIC can be differentiated by bleeding tendency, prolonged coagulation parameters and elevated fibrinogen degradation products (Shaikh, 2011).

The pathophysiology of HIT is complex, simply put, IgG is initiated against the platelet factor 4 (PF4)-heparin complex which subsequently activates platelets via FcyRIIa, propagated by activated platelets, endothelial cells, monocytes and coagulation proteins (McKenzie and Sachais, 2014). Platelet factor 4 is a cationic protein stored within platelet α-granules that is released upon platelet activation, this allows for the interaction with negatively charged GAGs, to form an immunogenic complex both in circulation and on the platelet surface (Arepally, 2017). These pathogenic PF4-heparin complexes are the target of HIT antibodies, these HIT antibodies attached to the PF4-heparin complex bind to platelet FcyRIIa, to activate platelets via intracellular signalling involving spleen tyrosine kinase and the release of procoagulant microparticles (Visentin, Ford, Scott and Aster, 1994). Thrombosis is propagated in this manner in a positive feedback loop, binding of PF4-heparin complexes to GAGs displaces bound antithrombin and renders the surrounding environment prothrombotic by release of procoagulation factors increasing the risk of thromboembolic events in HIT (Staibano, Arnold, Bowdish and Nazy, 2017).

Heparin-induced thrombocytopenia occurs in 3-5% of patients who receive intravenous unfractionated heparin compared to a 0.5% incidence rate with subcutaneous LMWH, catheter flushes and even small amounts of heparin leached from coated catheters (Kelton, 2002). Clinically HIT may develop in two distinct forms: type I and II (Baroletti & Goldhaber, 2006). HIT type I, is

a non-immunologic response to heparin treatment, mediated by a direct interaction between heparin and circulating platelets, resulting in platelet clumping or sequestration (Ahmed, *et al*., 2007). HIT type I occurs in up to 10% of patients and generally occurs within the first 48-72 hours following initiation of heparin treatment and is characterised by a mild and transient thrombocytopenia which often returns to normal in 4 days following withdrawal of heparin treatment (Franchini, 2005). Laboratory tests are not necessary to diagnose HIT type I, and is not associated with a risk of thrombosis, whereas HIT type II is immune-mediated and caused by an antibody against platelet factor 4 and associated with a risk of thrombosis (Amiral, *et al*., 1992). In 1-5% of cases, HIT can precede an extreme prothrombotic diathesis, HIT thrombosis, which results in venous or arterial thromboembolism in 50% of patients (Solanki, *et al*., 2019). Without prompt and effective treatment, limb amputation occurs in 10-20% of cases, death in 20-30% of cases and residual deficits in survivors that contribute to strokes, myocardial infarctions and pulmonary emboli (Almeida, *et al*., 1998).

The process of inflammation is an immunological response that can be triggered by a variety of factors, including damaged cells, pathogens, toxins, and trauma (Chen, *et al*., 2018). Infectious and non-infectious cell damage or disease activate inflammatory cells to trigger a number of inflammatory signalling pathways (Varela, *et al*., 2018). The main pathways include, NF-κB, MAPK and JAK-STAT and key events include leucocyte recruitment, adhesion, rolling and transmigration (Leick, *et al*., 2015). Molecules such as chemokines, integrins, selectins and enzyme modulate these processes, initial stages may be stimulated by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) depending on tissue type (Mogensen, 2009). Pathogen-associated molecular pattern molecules released from pathogens include lipopolysaccharides which act to stimulate the endothelium resulting in the upregulation of endothelial cell surface selectins such as P- and E-selectin (Panes, *et al*., 1999). Pathogen-associated molecular pattern molecules engage Toll like receptors (TLRs), to trigger a number of signalling pathways such as: NF-κB (Liu, *et al*., 2017), IFN regulatory factors (IRFs) (Kawai & Akira, 2007) and mitogen-activated protein kinases (MAPKs) (Rajgopal, *et al*., 2006). NF-κB and MAPKs have integral roles in the induction of proinflammatory responses (Park, *et al*., 2015) whereas IRFs are essential for stimulation of IFN production (Jeffries, 2019). Damage associated molecular patterns are released in response to tissue injury, autophagy, and cell death, including nucleic acids: mtDNA, cytosolic RNA, heat shock proteins and HA oligosaccharides (Land, 2015). These stimulate the innate immune system via the vasculature resulting in the extravasation of leucocytes into tissues (Nourshargh & Alon, 2014). Leucocyte and GAG expressed L-selectin, P selectin glycoprotein ligand (PSGL)-1 and P selectin are involved in the recruitment process by mediating the initial tethering and rolling (Huo & Xia, 2009).

Heparin binds to a number of proteins as shown in *Table 4*, including those involved in coagulation, growth factors, proteins involved in lipid metabolism and mediators of the immune system. Heparin binding protein (HBP) is a promising predictor of the acute phase in many critical diseases that activates both inflammatory and fiber-induced pathways (*Xue et al., 2022*). Acute phase protein blood plasma concentration levels increase or decrease in response to inflammation, these changes are largely due to changes in production by hepatocytes influenced by interleukins (IL) and TNF-α *(Jain, Gautam and Naseem, 2011)*. Positive acute phase proteins include C-reactive protein, ferritin, fibrinogen, hepcidin and serum amyloid A, whereas, negative acute phase proteins include albumin, prealbumin, transferrin, retinol binding protein and antithrombin (*Jain, Gautam and Naseem, 2011*). Recent research has demonstrated that low molecular weight heparin can ameliorate the acute phase response, decreasing serum concentrations of serum amyloid A and pro-inflammatory cytokines-TNF-α and modulating oxidative stress biomarkers (*Esmaeili Seraji, Chalmeh and Pourjafar, 2022*). Upon heparin binding, FGF changes conformation, this acts to protect FGFs from heat and acid mediated inactivation and nonenzymic glycosylation (Koledova *et al*., 2019). Fibroblast growth factors have been found to dimerise and oligomerise in the presence of heparin and heparin like GAGs to form a signalling complex (*Schlessinger et al., 2000*). Bioactivity of FGF-2 is dependent on the HSPG to which it is bound, when bound to glypican-1 FGF-2 sequestration prevents binding to FGFRs and signalling (Gutiérrez and Brandan, 2010), whereas when bound to an HSPG containing perlecan or syndecan-1 FGF-2 dependent signalling is enhanced (*Vincent et al., 2007*). Similarly, the cytokine interferon-gamma (IFN-γ), important for the induction and regulation of a number of immune responses binds to heparin (*Lortat-Jacob, Baltzer and Grimaud, 1996).* Heparin acts to protect the cytokine from proteolytic degradation by binding the C-terminal motif, blocking cleavage at the site which reduces clearance and increases it activity (*Lortat-Jacob, Baltzer and Grimaud, 1996*). The interaction of heparin with other cytokines is highly variable, and are more often inhibitory than augmentative, with regards to IL-2 there is no effect on bioactivity (*Najjam, 1998*), in contrast the effect of heparin on IL-3 induced proliferation of myeloid cells is concentration dependent, increasing at lower concentrations and suppressed at higher heparin concentrations (*Alvarez-Silva and Borojevic, 1996*). Similar to IFN-γ, heparin protects IL-7 from proteolytic degradation, however, IL-7 dependent pre-B cell growth is suppressed by heparin (Clarke *et al*., 1995). Heparin also prevents IL-10-induced expression of CD16 and CD64 on monocytes and macrophages (*Salek-Ardakani, Arrand, Shaw and Mackett, 2000*). More recently, heparin has been found to increase the localisation of IL-12 on cell surfaces, binding and signalling and was modestly protective against proteolytic degradation (*Jayanthi et al., 2017*).

Leukocyte adhesion and activation play a central role in the inflammatory response, with excessive activation resulting in intravascular aggregation and the release of reactive oxygen species and proteolytic enzymes that contribute to vascular and tissue damage (*Muller, 2013*). Evidence suggests that heparin may interfere with the adhesion of leukocytes to the endothelium. Early research demonstrated that in rats receiving unfractionated heparin or the synthetic analogue dextran sulphate leukocyte accumulation was reduced when compared to their untreated counterparts (*Yanaka et al., 1996*). More recently, the effects of UFH, LMWH and a selectively O-desulphated derivative of heparin were investigated for their effects on the adhesion of human polymorphonuclear leukocytes (PMNs) in vitro. All heparin preparations significantly inhibited the adhesion of f-met-leu-phe stimulated PMNs, similarly, pre-incubation with heparin followed by washing also evidenced the anti-adhesive effects even when not present in the system (*Lever, Hoult and Page, 2000*). To a lesser extent, heparin and partially desulphated derivatives are able to inhibit nonactivated neutrophils to platelet activating factor stimulated endothelial cells *vitro* (*Brown*, *Lever, Jones and Page, 2003*)

One of the earliest events in inflammation is the initial capture and rolling of neutrophils along the activated endothelium. Selectins mediate the loose interaction between leukocytes and endothelial cells, mediating rolling on vascular surfaces, responsible for the primary adhesive step during inflammation (*McEver, 2015*). The selectins are a family of three glycoprotein adhesion molecules: L-selectin, as expressed by leukocytes, P-selectin, expressed by both activated platelets and endothelial cells and E-selectin, expressed by activated endothelial cells (*Auvinen, Jalkanen and Salmi, 2014*). All three selectins are type I transmembrane proteins, which bind to carbohydrate-based ligands (*Vestweber and Blanks, 1999*). Sialyl Lewis[X] (sLeX), is considered the prototype counter ligand for selectins, structurally composed of a tetrasaccharide containing α1,3-fucose and α2,3-sialic acid (*Trinchera, Aronica and Dall'Olio, 2017).* Modifications such as sulphation of tyrosines and carbohydrates can improve the binding of P- and L-selectins to their ligands. Physiologically the counter-ligand for P-selectin is the sialomucin P-selectin glycoprotein ligand-1 (PSGL-1) expressed at the tips of leukocyte microvillae (*Pawar, Jadhav, Eggleton and Konstantopoulos, 2008*). In addition, PSGL-1 with CD44 is also a major leukocyte ligand for E-selectin (*Dimitroff et al., 2001*). Under static conditions, heparin and LMWH oligosaccharides have been shown to inhibit the binding of L-selectin and P-selectin chimeras to absorbed sLeX (*Nelson et al., 1993*). Furthermore, modified heparins have also been found to exhibit anti-adhesive properties in three human colon carcinoma cell lines under static and flow conditions, revealing a critical role for 6-*O*-sulphation of glucosamine units in heparin in the inhibition of P-selectin mediated adhesion (*Wei et al., 2004*). However *in vivo* investigations utilising non-anticoagulant *N*-acetyl-de-*O*-sulphated heparin demonstrated that oral treatment of two doses significantly inhibited eosinophil and neutrophil recruitment into the lungs (*Riffo-Vasquez et al.,*

2016). In addition, administration of platelets pre-treated with *N*-acetyl-de-*O*-sulphated heparin significantly reduced the numbers of leukocytes recruited to the lungs in response to bacterial lipopolysaccharide (*Riffo-Vasquez et al., 2016*). Similarly, unfractionated heparin and LMWH have been shown to inhibit neutrophil binding and aggregation on endothelial cell monolayers stimulated with platelet activating factor or thrombin, both of which induce P-selectin expression (*Brown, Lever, Jones and Page, 2003*). Passively or actively released elastase from neutrophils has been linked to a number of inflammatory diseases, including adult respiratory distress syndrome, heparin has been shown to inhibit the enzymatic activity and release of elastase *in vitro* (*Brown, Lever, Jones and Page, 2003*). Furthermore, various heparin preparations, UFH, LMWH and a selectively *O*-desulphated heparin lacking anticoagulant activity have been found to inhibit the adhesion of human polymorphonuclear lymphocytes to cultured human umbilical vein endothelial cells *in vitro* (*Lever, Hoult and Page, 2000*). This demonstrates that heparins anti-inflammatory effects can be in part attributed to the blocking of P- and L-selectin-initiated cell adhesion, structurally sulphation of C6 groups appears to be critical in selectin inhibition.

Following initial capture and rolling of neutrophils, the firm adhesion of neutrophils to endothelium is carried out by the leukocyte β_2 integrins LFA-1 and Mac-1, heterodimeric surface proteins and their endothelial counter receptor molecule, intercellular adhesion molecule-1 (ICAM-1) (*Li et al., 2013*). β_2 integrins are expressed on neutrophil cell surface and comprised of three distinct alpha chains: CD11a, CD11b and CD11c and a common β subunit, CD18 (*Schittenhelm, Hilkens and Morrison, 2017*). The four β_2 integrins have important roles in regulating immune processes including, recruitment to sites of inflammation, cell-cell contact formation and downstream effects on cellular signalling (*Schittenhelm, Hilkens and Morrison, 2017*). After binding, neutrophils can proceed by transendothelial migration into the interstitial space, a number of molecules have been implicated in stimulating this process, including PECAM-1, ICAM-2, CD99 and JAM-A (*Woodfin et al., 2007*). It has long been known that UFH and LMWH reduce the transmigration of polymorphonuclear neutrophil leukocytes through endothelial cell monolayers (*Hofbauer et al., 1999*). Immobilised heparin is known to mediate cell adhesion via interaction with the polymorphonuclear leukocyte integrin Mac-1 (*Diamond et al., 1995*). Heparin has also been shown to attenuate leukocyte rolling, adhesion and migration but does not affect expression of cell adhesion molecules or vascular permeability induced by TNF-α, further studies revealed that pre-incubation with an anti-CD11b monoclonal antibody not anti-CD11a or anti-L selectin antibody diminished heparin binding *ex vivo* (*Salas, 2000*). Furthermore, blocking antibodies to CD11b has been shown to inhibit heparin binding and as a result abolishes the apoptotic response and decreases the rate of superoxide release from polymorphonuclear leukocytes (*Cohen-Mazor et al., 2015*). This suggests that the anti-inflammatory effects of heparin could be related to the attenuation of a CD11b dependent adherent mechanism.

Molecular studies have determined that heparin and related compounds may exert anti-inflammatory effects through the transcription factor, nuclear factor kappa B (NF-κB). The NF-κB pathway has long been known as a prototypical pro-inflammatory signalling pathway, based on the activation of NF-κB by proinflammatory cytokines such as IL-1 and TNF-α and the role of NF-κB in the expression of other proinflammatory genes including cytokines, chemokines and adhesion molecules (*Mussbacher et al., 2019*). In most unstimulated cells, NF-κB dimers comprised of NF-κB and the inhibitory protein IκB are sequestered in an inactive form in the cytosol (*Smale, 2012*). Upon stimulation, the NF-κB-IκB complex is degraded via phosphorylation by the IκB kinase, exposing a highly cationic domain of 9 amino acids to result in the nuclear translocation of NF-κB and induction of transcription target genes (*Solt and May, 2008*). Several studies have demonstrated that heparin can be bound and internalised into the cytosolic compartment of the endothelium, vascular smooth muscle cells, liver cells and cardiac myocytes (*Olofsson et al., 1999*) (*Letourneur, Caleb and Castellot, 1995*) (*Urano, Haba, Yuasa and Watanabe, 1997*). Following internalisation, heparin can bind electrostatically to the cationic NF-κB inhibiting NF-κB DNA-binding activity without affecting degradation or nuclear translocation (*Lee et al., 2008*). Both heparin and 2,3-*O*-desulphated heparin binding were found to inhibit the expression of adhesion molecules such as ICAM-1 and vascular cell adhesion molecule (VCAM-1) (*Lee et al., 2008*). This is supported by studies of bEnd.3 cells, whereby heparin was found to inhibit both TNF-α and oxygen-glucose deprived induced NF-κB activation (*Lee et al., 2007*). Ultimately suggesting that internalisation and binding of heparin to NF-κB prevents the initiation of inflammatory gene activation and regulates the gene expression and production of proinflammatory cytokines, chemokines and adhesion molecules. Independent of its anticoagulant activity, heparin and *O*-desulphated heparin have been demonstrated to reduce inflammation following myocardial reperfusion injury. Data demonstrates that both heparin and *O*-desulphated heparin inhibited NF-κB translocation and reduced NF-κB DNA binding in human endothelium and ischemic-reperfused rat myocardium (*Thourani et al., 2000*). In addition, both heparin and *O*-desulphated heparin equally reduced neutrophil adherence to ischemic-reperfused myocardium, influx of neutrophils, myocardial necrosis, and release of creatine kinase into the plasma (*Thourani et al., 2000*). Similarly, low molecular weight heparin has been found to have anti-inflammatory potential, pre-treatment of valvular endothelial cells with enoxaparin has been found to inhibit TNF-α induced ICAM-1 expression and lipopolysaccharide-induced E-selectin expression, acting to reduce monocyte adhesion (*Manduteanu et al., 2002*). Both unfractionated heparin and LMWH demonstrate equal ability to significantly reduce the monocytic inflammatory reaction via the inhibition of NF-κB activation to reduce levels of TNF-α, CXCL8, IL-6 and IL-1β and nuclear translocation (*Hochart, Jenkins and White, 2005*). More recently, rat models of inflammation in the upper respiratory tract, low molecular weight heparin has been shown to

inhibit the activation of the TLR4-MyD88- NF-κB signalling pathway and the subsequent down-regulation of inflammatory factor gene expression (*Wu et al., 2021*).

A number of studies have demonstrated the modulatory activity of heparin on TNF-α and NF-κB, with the primary mode of action being inhibition of nuclear translocation, and suppression of TNF-α-induced and NF-κB-mediated secretion and expression of CXCL-8 and IL-6 (*Spratte et al., 2012*). Both TNF-α and NF-κB are members of the apoptosis cascade involved in cell death, indicating a therapeutic potential in this area. Activation of apoptosis and programmed cell death can be initiated by a number of intrinsic cues or activation of relevant pathways by binding of specific protein ligands to the cell surface (*Lall, 2009*). A significant receptor of importance is TNFR1 (p55), a member of the tumour necrosis factor family. TNFR1 is a death receptor, containing a death domain in its cytoplasmic part, this allows homotypical interaction with other cytoplasmic proteins containing a death domain (*Park et al., 2007*). Death domain containing signalling proteins link TNFR1 to cytotoxic signalling pathways to trigger apoptosis or necroptosis, but also allow the engagement of other signalling pathways activating transcription factors of NF-κB or members of the MAP kinase family (*Brenner, Blaser and Mak, 2015*). Upon TNF-α binding, TNFR1 binds death domain-containing cytoplasmic proteins TNFR1-associated death domain (TRADD) and receptor interacting protein kinase-1 (RIPK1) to form a trimer that can bind a series of other proteins (*Füllsack, Rosenthal, Wajant and Siegmund, 2019)*. TNFR1-bound TRADD and RIPK1 recruit TNFR-associated protein-2 and Fas-associated death domain proteins to ultimately activate caspase-8 and initiate the entire cascade of other caspases to initiate and execute apoptosis (*Brenner, Blaser and Mak, 2015*). Heparin has the potential to intervene in the early stages of this pathway, by reducing TNF-α available to induce TNFR1 death domain mediated apoptosis. Studies have demonstrated that heparin can inhibit gene expression and production of TNF-α in ischemic rat heart and block p-selectin and integrin mediated recruitment of neutrophils- both of which are rich sources of TNF-α production (*Thourani et al., 2000*). This not only inhibits the TNFR1 mediated pathway of apoptosis, but has effects downstream the caspase cascade, as the TNFR1 and mitochondria mediated pathways converge on a common downstream pathway at effector caspases such as caspase 3 (*Cullen and Martin, 2009*).

The intrinsic pathway of apoptosis is characterised by the initiation of caspase activity through the mitochondria. Most stimuli induce apoptosis via this pathway, the defining event of which is mitochondrial outer membrane permeabilization (MOMP) to release intermembrane space proteins into the cytosol (*Kalkavan and Green, 2017*). Notably, cytochrome *c*, is released into the cytosol, in mitochondrial ATP generation cytochrome *c* acts to shuttle electrons between complexes III and IV of the electron transport chain (*Hüttemann et al., 2011*). However, when released from the mitochondria, cytochrome *c* adopts a deadly function essential for caspase activation (*Jiang and Wang, 2000*). Positively charged cytochrome *c* binds the adaptor molecule

APAF-1 to induce the conformational changes leading to oligomerisation and the formation of a heptameric polymer known as an apoptosome (*Yuan et al., 2013*). In turn, this apoptosome recruits and activates pro-caspase-9 which then cleaves and activates caspases -3 and -7 (*Jiang and Wang, 2000*). As a strong polyanion it was postulated that heparin may compete with APAF-1 to bind positively charged cytochrome *c* to protect the cell from apoptosis. The electrostatic interaction between heparin and proteins has been shown to have inhibitory effects on the activity of other positively charged granular neutrophil proteases such as human leukocyte elastase and cathepsin G (Koster *et al*., 2002) (Ermolieff *et al*., 1994). Differential scanning calorimetry determined that heparin binding at low ionic strength induced a significant shift of the transition temperature of cytochrome *c* (Bágel'ová, Antalík and Bona, 1994). Despite this there is no recent evidence to show that heparin can directly bind to and inhibit cytochrome *c*. Physiologically, heat shock protein 27 (Hsp27) acts as a protein chaperone, an antioxidant and has a role in the inhibition of apoptosis and actin cytoskeleton remodelling (*Vidyasagar, Wilson and Djamali, 2012*). Specifically, Hsp27 binds to cytochrome *c* released from the mitochondria to prevent the cytochrome *c*-mediated interaction of APAF-1 with procaspase-9 to prevent mitochondrial dependent apoptosis and caspase mediated apoptosis (*Bruey et al., 2000*) (*Beere, 2001*). Heparin-like molecules have been found to stimulate low levels of Fas-mediated apoptosis in T lymphocytes, to decrease Fas receptor aggregation and ultimately abolish the anti-apoptotic effects of Hsp27 (Manero *et al*., 2004).

There is evidence that heparin may act to modulate the activity of a number of other pro-apoptotic proteins. The ability of heparin to activate multiple anti-apoptotic pathways has been demonstrated in human trophoblasts (*Hills et al., 2006*). Heparin was shown to abrogate apoptosis of primary first trimester villous trophoblasts in response to treatment with pro-inflammatory cytokines interferon (IFN)-γ and TNF-α, other agents including: staurosporin, broad-spectrum kinase inhibitor and thrombin (*Hills et al., 2006*). Specifically, it was demonstrated that heparin can elicit the phosphorylation of EGFR and activation of the phosphatidyl inositol 3-kinase (PI3K), extracellular signal-related kinase 1/2 (ERK1/2) and the c-Jun NH2 terminal kinase (JNK)-signal transduction pathways in primary villous trophoblasts (*Hills et al., 2006*). The major apoptotic nuclease, DFF40/CAD specific for double stranded DNA can be inhibited by heparin, mechanistically this is proposed to be a result of heparin binding to the positively charged surface formed by α helices of the DFF40/CAD homodimer (*Widlak and Garrard, 2006*). It has been demonstrated that phagocytic clearance of apoptotic cells induces active anti-inflammatory and immunosuppressive responses, it is thought that this process can be augmented by heparin treatment (*Yun, Henson and Tuder, 2008*). High affinity binding sites for heparin have been found to be generated on leukocytes during apoptosis, which when blocked by a highly potent inhibitor of heparin binding, pentosan polysulphate, inhibits phagocytosis (*Gebska, 2002*). This

demonstrates that heparin binding may have a significant role in the clearance mechanism of dead cells, and the subsequent anti-inflammatory responses (*Gebska, 2002*).

The potential of heparin as an anti-inflammatory thus far has been curtailed by its potent anticoagulatory activity. However, a number of clinical trials modelling inflammatory diseases has supported the anti-inflammatory effects of heparin with little to no adverse anticoagulatory effects. This activity is most exemplified by studies of asthma symptoms, with the most promise being shown in trials utilising nebulised heparin. It has long been known that inhaled heparin attenuates the broncho-constrictive response in patients with exercise induced asthma (*Ahmed, Garrigo and Danta, 1993*). Further studies using inhaled enoxaparin showed similar protective effects and was superior to unfractionated heparin at higher doses (*Shastri et al., 2015*). Mechanistically, low molecular weight fractions of enoxaparin inhibited the release of IL-4, IL-5, IL-13 and TNF-α (*Shastri et al., 2015*). More recently, sulphated non-anticoagulant heparin administered via intraperitoneal injection was shown to reduce airway eosinophilia, mucus production and airway hyperresponsiveness following chronic repeated models of asthma (*Ghonim et al., 2018*). Similarly, it was found that these affects could be attributed to the suppression of IL-4, IL-5, IL-13, granulocytes macrophage colony-stimulating factor (GM-CSF), and ovalbumin IgE with no effect on IFN-γ. However, nebulised heparin as a treatment for bronchoconstriction in response to bronchostimulants has mixed results. In response to AMP, inhaled UFH successfully attenuates airway responsiveness, with a peak at 15 minutes lasting for up to an hour, whereas in response to methacholine UFH is not effective at attenuating the response (*Polosa et al., 1997*). This suggested a protective role of heparin against AMP provocation by inhibiting the activation of mast cells, supported later by further research (*Zeng et al., 2004*).

In response to allergen induced asthma, the effectiveness of heparin appears to be time, dose, and administration dependent. Single doses of inhaled heparin in animal studies revealed a slight reduction in the early asthmatic response (EAR) but had little effect on the late asthmatic response (LAR) in response to dust mite extract (*Diamant et al., 1996*).. However, extending the dosage and prophylactically and therapeutically administering inhaled UFH demonstrated a slight but not significant reduction in the EAR but a statistically significant reduction in the LAR when compared to the placebo (*Diamant et al., 1996*). More recently, a similar small-scale murine model of allergic airway inflammation demonstrated that heparin and low molecular weight heparin when administered intranasally had a protective effect against inflammation (*Fu et al., 2013*). Case studies of two corticosteroid resistant patients demonstrated that nebulised unfractionated heparin could act to ameliorate symptoms during exacerbation, with no adverse coagulation effects (*Bendstrup and Jensen, 2000*).

Although primarily used as an anticoagulant, the processes that heparin influences have some overlap with inflammatory pathways (Urban, Gordon, Farrell and Shaffer, 1991). Heparin not only displays anticoagulant activity by inactivation of thrombin and FXa but also impacts inflammatory processes, such as the release of inflammatory cytokines by activated platelets and thrombin activated monocytes (*Esmon, 2004*).The use of heparinised extracorporeal circuits as in a cardiopulmonary bypass has been shown to reduce inflammatory mediators from the complement cascade, granulocytes and cytokine release specifically suppressing IL-6 whilst upregulating IL-10, an anti-inflammatory cytokine (*Harig et al., 1999*). Further research demonstrated that heparin concentration-based anticoagulation management, when compared to activated clotting time heparin more effectively attenuates the inflammatory response. The use of concentration-based anticoagulation reduced neutrophil activation, demonstrated by reduced neutrophil elastase levels (*Koster et al., 2002*). Furthermore, data showed a trend toward lower C5b-9 values, an activator of the terminal complement pathway, indicating heparin may act to suppress complement activation in this manner (*Koster et al., 2002*). Despite promising results, heparin is currently only clinically used for the prevention of thrombosis in the circuits used for extracorporeal circulation (*Delavenne et al., 2017*).

Other potential applications of the anti-inflammatory properties of heparin include inflammatory bowel diseases (IBD), cardiopulmonary bypass and in the treatment of burns. The most studied IBD in relation to heparin treatment is ulcerative colitis, the evidence for the clinical application of heparin is conflicting. A small prospective randomised study revealed that whilst the use of enoxaparin had no adverse effects and was well tolerated, there was no additive benefit over standard therapy, with no significant differences in fibrinogen, erythrocyte sedimentation rate (ESR) and C-reactive protein values between control and enoxaparin group (*Zezos et al., 2006*). A recent study utilising extended colon-release tablets as opposed to subcutaneous administration found a statistically significant benefit for low molecular weight heparin when compared to the placebo (*Lean et al., 2015*). The parameters for success included reaching clinical remission and improvement, endoscopic improvement and reduced rectal bleeding (*Lean et al., 2015*).

Though widely used for its anticoagulant properties, heparin has been known to display other properties that may have conferred clinical benefits in clinical trials. In light of the coronavirus-19 pandemic, a large amount of time and resources was directed towards the repurposing of available drugs, resulting in an influx of research pertaining to heparins anti-inflammatory properties. The physiological location of heparin, in mast cells, suggests a role in inflammation, mast cells are immune cells with roles in the regulation of vasodilation, vascular homeostasis, innate and adaptive immune responses, angiogenesis and venom detoxification. Potential mechanisms of anti-inflammatory action include binding to different mediators- cytokines, chemokines, acute phase proteins and complement complex proteins, neutralising cytokines at the site of

inflammation, inhibition of leukocyte and neutrophil adhesion, the inhibition of nuclear factor κB and induction of apoptosis by modulating the activity of TNF-α and NF-κB. Despite this, it has not been approved for clinical usage due to its structural diversity and ability to disrupt many physiological processes (*Lakshmi, et al., 2010*). More thorough research needs to be conducted to determine the mechanisms by which heparin effects the inflammatory response, to identify key targets in the numerous pathways involved, to determine the specific structural requirements of heparin and heparin analogues that support anti-inflammatory action and abrogate the anticoagulant activity and the optimal dosage and mode of administration.

2.4. The coagulation cascade

One of the most prominent and well researched applications of heparan sulphate and heparin, is the involvement of these GAGs in the coagulation cascade. Heparan sulphate and heparin can bind to antithrombin III, resulting in the inhibition of thrombin and factor Xa (FXa) (Conard, *et al.*, 1983) (Pike, *et al.*, 2005). Antithrombin (AT) is a major serpin anticoagulant, it is a ~58kDA glycoprotein circulating in the blood that can inhibit a number of serine proteases involved in coagulation (Roemisch, *et al.*, 2002). Serpins are a broadly distributed family of protease inhibitors that when conformationally changed, inhibit target enzymes, in the case of ATIII, these enzymes are thrombin and FXa (Law, *et al.*, 2006). Upon heparin binding at the D-helix, the structure of AT changes, expelling the reactive centre loop (RCL), this conformational change is thought to accelerate the interaction of AT with serine proteases such as FXa but not enzymes such as thrombin (Munoz & Linhardt, 2004) (Jin, *et al.*, 1997). This increase in interaction with FXa is thought to be caused by the expelling of the exposed reactive centre loop (RCL) allowing for interaction of its residues with subsites on the active site of FXa (Quinsey, *et al.*, 2002) (Rezaie, *et al.*, 2004) and the exposure of an exosite on the serpin, with residues including Arg 46, Arg47, Lys114, Lys125 and Arg 129 that can bind to heparin (Olson, *et al.*, 2010) (Richard, *et al.*, 2018). Heparin binding is mediated by heparin pentasaccharide-mediated conformational change, conferred by 3-*O*-sulphation of glucosamine (GlcNS6S) (Richard, *et al.*, 2018).
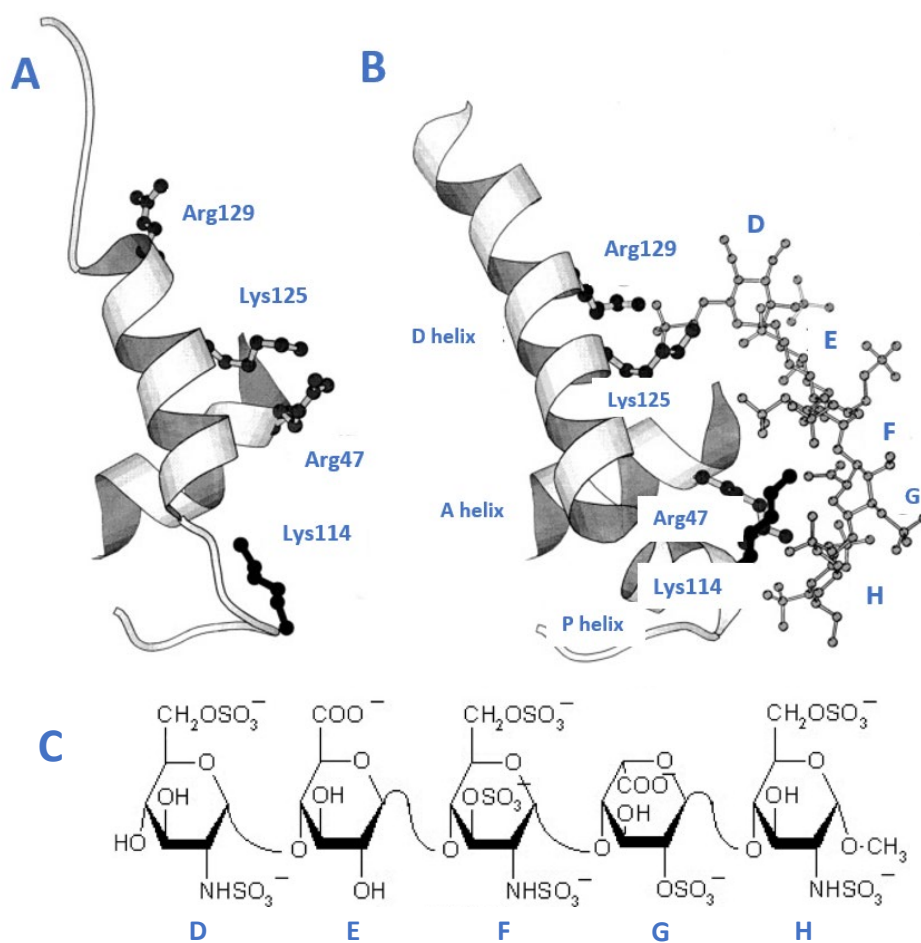
Figure 5: Conformational change of antithrombin upon heparin binding. *A: without pentasaccharide. B: with bound pentasaccharide. The side chain of Lys114 is shown in black,*

whereas the side chains of other major pentasacchairde binding residues, Arg47, Lys125 and Arg129 are shown in dark grey. The pentasaccharide, DEFGH is displayed in light grey, structures from (Skinner, et al., 1997). C: pentasaccharide binding sequence, adapted from (Desai, et al., 2000).

Only one third of heparin chains, possess the unique pentasaccharide sequence that confers anticoagulant activity by binding to antithrombin (Andersson, *et al*., 1976). In higher doses, heparin chains with or without this sequence can activate heparin cofactor II, a second plasma cofactor (Tollefsen, *et al*., 1981). Later work has provided evidence that perhaps the 3-O-sulphate is not required for heparin-antithrombin binding, that the identification of this sequence was an artefactual finding, due to the experimental conditions utilised (HajMohammadi, *et al*., 2003). Further, this pentasaccharide sequence has yet to be identified within HS, one of the physiological modulators of AT binding (Meneghetti, *et al*., 2015). This is not to disregard the affinity of the pentasaccharide sequence as identified in *Figure 5,* but to demonstrate the difference between the pharmacological action of heparin and the physiological activity of heparan sulphate. A more complex understanding of antithrombin activation is becoming more widely accepted, as opposed to the previous lock and key understanding, whereby the net charge of a ligand has a significant role in the affinity of heparin to AT (Seyrek, *et al*., 2007). This is supported by increasing bodies of work demonstrating that other carbohydrate structures, unalike GAGs, can bind AT, but have negligible anticoagulant activity (Chavante, *et al*., 2014). Further research has shown that modifications, like N-acetylation and N-sulphation adjacent to, within, and outside of the first glucosamine residue of the pentasaccharide can induced antithrombin binding. (Guerrini, *et al*., 2012). Data has shown a possible role for the non-sulphated Iduronic acid that precedes the pentasaccharide sequence, with conformational data demonstrating the adoption of the $^2S_0$ conformation in the presence of AT, whereas, when AT is absent a $^1C_4$ form is predominantly adopted (Guerrini, *et al*., 2006). Another study, (Guerrini, *et al*., 2008) confirms this finding, with the extension of the pentasaccharide chain by non-sulphated IdoA increases the affinity of AT binding. Perhaps most interestingly, this study showed the increase in magnitude of affinity for AT binding, increasing by the order of 1 when a GlcA residue precedes the pentasaccharide sequence (Guerrini, *et al*., 2008).

Heparin/AT complexes inactivate thrombin (factor IIa) and factors Xa, IXa, XIa and XIIa acting to inhibit the intrinsic pathway of coagulation and the final stages of coagulation regardless of intrinsic or extrinsic pathway, as seen in *Figure 5* (Merlini, *et al*., 1994). Heparin catalyses inhibition of thrombin by antithrombin, by simultaneously binding to both antithrombin at the pentasaccharide sequence and thrombin in a non-specific charge-dependent manner (Izaguirre, *et al*., 2014). Formation of this ternary-AT-thrombin complex bridges the inhibitor and enzyme together can only occur with heparin chains of 18 or more saccharide units (De Caterina, *et al*., 2013). However, shorter pentasaccharide-containing heparin molecules can catalyse FXa inhibition by only binding

AT, as this reaction does not require bridging (Garcia, *et al.*, 2012) (Casu, *et al.*, 1981). In both cases, binding occurs at a rare 3-*O*-sulphation of glucosamine (GlcNS6S) (Thacker, *et al.*, 2013).

To some extent, heparin can also catalyse AT-mediated inhibition of other coagulation factors, including FVIIa, FIXa, FXIa and FXIIa and has other anticoagulant properties (Al-Horani & Afosah, 2018). Heparin can inhibit the proteolytic function of FXIa, directly or indirectly, ultimately acting to inhibit the primary function of FXIa- to activate FIX to FXIa which results in thrombin generation. Direct inhibition can occur either by charge neutralisation or allosteric modulation (Al-Horani & Desai, 2014). This modulation occurs by binding to a select group of basic amino acids in the catalytic domain of FXIa: Lys529. Arg530, Arg532, Lys535 and Lys539 (Yang, *et al.*, 2009). Indirect inhibition occurs the formation of an inhibitory ternary complex by which the A3 domain of FXIa (Lys252, Lys253 and Lys255) and the serpin bind to the same sequence of heparin (Yang, *et al.*, 2009). Heparin treatment has been found to reduce the activity of FVIIa by 50% following a bolus injection followed by continuous infusion of heparin for 24 hours (Hansen, *et al.*, 2000). It is thought that heparin decreases the proteolysis of FVII to FVIIa by antithrombin or enhances the inactivation of FXa by tissue factor pathway inhibitor (TFPI) (Perenrstorfer, *et al.*, 2005). Heparin-induced release of TFPI would certainly support the latter (Hansen, *et al.*, 2001). Heparin inhibits FXIIa, by inhibiting the classic complement pathway (Rent, *et al.*, 1976). This system is initiated when FXII binds to negatively charged surfaces, to become activated, FXIIa (Muller, *et al.*, 2011). Upon activation FXIIa can cleave FXI of the intrinsic coagulation pathway into FXIa which triggers thrombin formation and clotting (Gailani & Renne, 2007). Not only this but FXIIa can cleave prekallikrein into kallikrein, which releases bradykinin upon cleavage of kinogen by kallikrein, causing vasodilation (Sainz, *et al.*, 2007). FXIIa can be cleaved by kallikrein into Hageman factor fragment, which can enzymatically activate the primary component of the classic complement pathway (Sainz, *et al.*, 2007). Heparin potentiates the action of human C1-inhibitor (C1-INH), a naturally occurring serine protease inhibitor that inhibits the activation of the classic complement pathway (Rajabi, *et al.*, 2012).
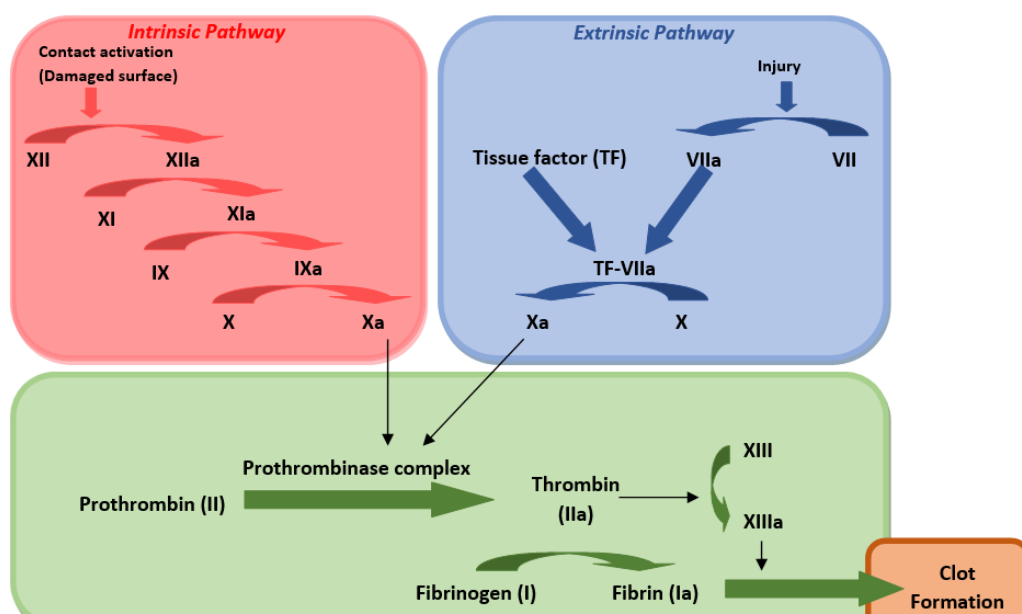
Like unfractionated heparin (UFH), LMWH can exert anticoagulant effects by activation of AT and accelerates the rate at which Factor Xa and thrombin are inhibited (Alquwaizani, *et al*., 2013). As only pentasaccharide-containing chains consisting of at least 18 saccharides are sufficient to bridge AT to thrombin, at least 50-75% of low molecular weight heparin (LMWH) chains are too short to catalyse thrombin inhibition by AT but retain the ability to promote Factor Xa inhibition (De Caterina, *et al*., 2007). As a consequence, LMWH preparations have a greater capacity to promote FXa inhibition than thrombin inhibition with anti-FXa to anti-FIIa activity ratios ranging from ~2:1 to ~4:1 depending on their molecular weight profiles (Patrono, *et al*., 2004). Whereas, UFH has an anti-FXa to anti-FIIa ratio of 1 (Libby, 2004). Unfractionated heparin also stimulates the release of platelet factor-4 (PF-4) from platelets, a heparin-PF-4 complex can form, responsible for causing heparin-induced thrombocytopenia (HIT) type 2 following heparin administration (Ahmed, *et al*., 2007). Inactivation of thrombin or reducing its generation by heparin, not only prevents fibrin formation but inhibits thrombin-mediated feedback of FV, FVIII and FXI and attenuates thrombin-induced aggregation of platelets (Bates & Weitz, 2005).

2.5. Uses of heparin as inhibitors or potential treatments of viral infection

Given that GAGs are ubiquitous on the surface of cells, it is not surprising that they serve as broad spectrum and non-specific receptors for virus binding (De Pasquale, *et al*., 2021). The interactions are thought to be generally based on electrostatic interactions between negatively charged GAGs and positively charged amino acid sequences on the viral envelope proteins (Lima, *et al*., 2017). Glycosaminoglycans have been implicated in the binding of many viruses to cell surfaces including, flaviviruses such as dengue virus (DENV) (Lin, *et al*., 2002), Japanese encephalitis virus (JEV) (Yun & Lee, 2018) and Zika virus (ZIKV) (Ghezzi, *et al*., 2017), filoviruses (Salvador, *et al*., 2013), hepatitis B and C viruses (Zahn & Allain, 2005), HIV (Bugatti, *et al*., 2019), influenza strain H5N1 (Skidmore, *et al*., 2015), measles virus (Terao-Muto, *et al*., 2008).

Host cell surface proteoglycans, bearing heparan sulphate and chondroitin sulphate are involved in initial flavivirus attachment, including DENV (Dalrymple & Mackow, 2011) (Artpradit, *et al*., 2014), JEV (Liu, *et al*., 2004) (Chen, *et al*., 2010), tick borne encephalitis (TBE) (Kroschewski, *et al*., 2003) (Kozlovskaya, *et al*., 2010) and YFV (Germi, *et al*., 2002). The precise mechanism by which dengue infection leads to fatal haemorrhagic fever and shock syndrome is not precisely understood (Dalrymple & Mackow, 2011). However, it is thought the major symptoms of severe dengue, vascular leakage and injury can be attributed to the accumulation of the secreted dengue NS1 glycoprotein on microvascular endothelial cells (Puerta-Guardo, *et al*., 2016). It has been

shown that inhibition of integrins and other potential cellular receptors was unable to reduce dengue infection of human endothelial cells (Dalrymple & Mackow, 2011). Interestingly, pre-treatment of endothelial cells with heparinase III or addition of heparan sulphate or heparin significantly inhibited dengue infection and dengue virions bound specifically to heparin affinity resins (Dalrymple & Mackow, 2011) (Vervaeke, *et al*., 2013). Therefore, it is thought that dengue virus appears to use cell surface heparan sulphate for several purposes including attachment, entry, and induction of immune-mediated endothelial damage through the accumulation of secreted NS1 glycoprotein (Dalrymple & Mackow, 2011). Japanese encephalitis virus infects a broad range of cell types *in vitro*, however, like DENV the initial events of JEV infection have not yet been fully elucidated (Yun & Lee, 2018). It has been found that highly sulphated GAGs are involved in the infection of both neurovirulent and attenuated JEV strains (Su, *et al*., 2001). Competition experiments using highly sulphated GAGs heparin and dextran sulphate, demonstrated inhibition of JEV attachment and infection of BHK-21 cells (Su, *et al*., 2001). Similar to DENV, heparin supressed the cytopathic effects induced by JEV infection in cultured cells (Su, *et al*., 2001). Level of sulphation has also been found to be of great importance, treatment of target cells by a potent sulphation inhibitor greatly reduced viral binding ability and infection (Su, *et al*., 2001). In the case of TBE, it has been reported that HS can function as an attachment receptor for both mutant and wild-type virus, but the affinity of this is significantly higher in mutant cells (Kroschewski, *et al*., 2003). However, mutant Chinese hamster ovary (CHO) cells deficient in HS synthesis have been found to be capable of binding much less virus but were still highly susceptible to infection by both mutant and wild-type TBE, suggesting another host-cell receptor that can mediate TBE viral entry (Kroschewski, *et al*., 2003). The role of heparan sulphate in the viral attachment and internalisation of ZIKV is still unclear (Gao, *et al*., 2019). Heparan sulphate deficient cells maintain the same levels of attachment and internalisation as unmodified cells, however, the early RNA and protein levels of ZIKV are impaired in HS deficient cells (Gao, *et al*., 2019). Furthermore, HS promotes cell death induced by virus infection and inhibition of cell death significantly increased the viral replication of ZIKV (Gao, *et al*., 2019). Heparin has been found to protect infected human neural progenitor cells from ZIKV induced cytopathic effects, preventing cell death, with slight inhibition of infectivity (Ghezzi, *et al*., 2017). Furthermore, the potential neuroprotective effects of heparin have been further demonstrated with roles in prevention of ZIKV-induced intracellular vacuoles, a defining feature of programmed cell death (paraptosis) and necrosis and apoptosis of human neural progenitor cells when grown as neurospheres (Pagani, *et al*., 2021).

Filoviruses are among the most virulent pathogens affecting humans, causing viral haemorrhagic fever (Kuhn, 2008), the most well-known filoviruses are Ebola virus and Marburg virus (Emanuel, *et al*., 2018). It has been demonstrated that filoviruses utilise GAGs, specifically heparan sulphate proteoglycans for host cell attachment (Salvador, *et al*., 2013). Hepatitis B (HBV) and C (HBC)

infections have been found to be mediated by heparan sulphate proteoglycans (Sureau & Salisse, 2013) (Xu, *et al*., 2015). It has been found that HBV and HCV from chronically infected patients bind to heparin (Zahn & Allain, 2005). In the case of HBV infection was abrogated by incubation of virions with heparin (Schulze, *et al*., 2007). Further, enzymatic removal of defined acidic carbohydrate structures from the cell surface using heparinase I/III or the obstruction of GAG synthesis by sodium chlorate inhibited HBV infection of HepaRG cells and lead to the reduction of HBV binding sites (Schulze, *et al*., 2007). Heparin has also been found to inhibit HBV infection in primary Tupaia hepatocyte cultures *in vitro*, pre-treatment of these cells with heparinase decreased viral binding and inhibited HBV infection completely (Leistner, *et al*., 2007). Similarly, in the case of HCV, heparin and liver-derived highly sulphated heparan sulphate inhibited cellular binding and entry of virus-like particles in a dose-dependent manner (Barth, *et al*., 2003).

The role of cell surface proteoglycans in human immunodeficiency virus (HIV), particularly syndecans has long been known (Patel, *et al*., 1993). The role of the HSPGs syndecans in viral binding has been validated by the lack of binding in syndecan deficient K562 erythromyeloblastoid cells, yet attachment does occur when K562 cells are transfected with syndecan-1 (Saphire, *et al*., 2001). Syndecan-2, syndecan-4 and CD44v3 expressed on human activated CD4+ T cells have been found to bind to HIV-1 p17 matrix proteins (De Francesco, *et al*., 2011). Cd44v3 is a member of the CD44 family of cell surface expressed proteoglycans with structural heterogeneity generated by the alternative splicing of at least nine exons in humans encoding membrane-proximal domains of the extracellular region (Screaton, *et al*., 1992). The v3 exon site is the only exon that has a heparan sulphate assembly site (Greenfield, *et al*., 1999). It has been shown that the cross-linking of HS side chains of syndecan-2 and syndecan-4 on activated naïve and memory CD4+ T cells inhibited cell proliferation and TNF-α production (Teixe, *et al*., 2008). It has been shown that the binding of dendritic cell associated HSPG-dependent integrin ligand to HS chains of syndecan-4 on activated T cells results in a down-regulation of proinflammatory cytokines and inhibited cell proliferation by blocking the S phase of the cycle (Chung, *et al*., 2009). This binding pathway could be manipulated to treat T-cell driven disorders, like HIV-1. (Chung, *et al*., 2009). On dendritic cells it is thought that syndecan-2 and syndecan-3 are the major HIV receptors, it has been shown that dendritic cells exploit syndecan-3 and DC-SIGN on dendritic cells to mediate HIV-1 transmission, promoting transmission to CD4+ cells and prolonging the infectivity of HIV-1 (de Witte, *et al*., 2007). Investigations including cytopathic effect assay, quantification of reverse transcriptase production and syncytia formation, have demonstrated that enzymatic treatment of HIV-1 susceptible lymphoblastic T-cell lines, MT-4 and H9, with heparinase significantly prevented HIV-1 infection (Patel, *et al*., 1993). Quantification of direct virus binding to cells showed that heparinase treatment of cells inhibited HIV-1 binding to the surface of T-cells as did the addition of exogenous heparan sulphate (Patel, *et al*., 1993). Further research has identified that the HIV-1

transactivating factor Tat accumulates on the surface of endothelium by interaction with HSPGs (Urbinati, *et al*., 2009). Heparin binds extracellular HIV-1 Tat protein and modulates its HIV long terminal repeat (LTR)-transactivating activity, binding with heparin and heparan sulphate immobilises gluthathione S-transferase (GST) Tat and inhibits HIV-LTR transactivation induced by extracellular GST-Tat (Rusnati, *et al*., 1997). A very small pilot trial determined that low-molecular weight heparin, enoxaparin, given in standard prophylactic doses stabilised or increased CD4 counts in patients in the first 3 months of treatment, then CD4 counts remained stable or decreased after 6 months (Howell, *et al*., 1996). Despite this promising early research, the feasibility of heparin as a potential adjunct therapy has been diminished by the proven efficacy of antiretroviral treatment (ART) that is still the standard since the first licensed use in 1987 (Richman, 1990). Furthermore, later investiagtions demonstrated that HIV infection increases the risk of HIT, meaning that at this point, the administration of heparin as an adjunct treatment for HIV is not feasible (Thompson, *et al*., 2007).

The inhibitory activity of heparin has also been demonstrated in the case of influenza, strain H5N1, with recombinant HIV-1 pNL4-3 vectors devoid of the HIV envelope gene with the HA gene from an avian H5N1 isolate (Skidmore, *et al*., 2015). Heparin and dextran sulphate have been found to be significantly inhibit viral invasion of H5N1 in 293T cells (Skidmore, *et al*., 2015). Further, it was shown that a reduction in sulphation levels resulted in more potent inhibition of invasion than seen with parental porcine mucosal heparin (Skidmore, *et al*., 2015). The decrease in sulphation levels also ameliorated the most significant adverse effects of heparin, the anticoagulant properties (Skidmore, *et al*., 2015). These results are promising as they provide a basis for research using modified heparins for use as antivirals. Similarly, HSPGs have been found to have a role in the infection of morbilliviruses *in vitro* (Baron, 2005) (Fujita, *et al*., 2007), such as measles, with implications in the entry of measles virus into cells (Terao-Muto, *et al*., 2008). The primary receptor of measles is the signalling lymphocyte activation molecule (SLAM); however, other receptors are being explored. Heparin has been found to prevent measles virus infection in cell lines by binding the envelope H glycoprotein- haemagglutinin (Terao-Muto, *et al*., 2008),

Heparan sulphate proteoglycans have been found to be important in the entry of many viruses, with many studies displaying the inhibitory properties of heparin (Cagno, *et al*., 2019). The repurposing of heparin as an antiviral is of interest as the safe clinical dosages have already been determined, it is relatively easy to access and has a broad range of antiviral activity against many enveloped viruses and has anti-inflammatory activity (Conzelmann, *et al*., 2020). Further research can be carried out to determine a modified form of heparin or synthetic analogue that may have little to no anticoagulant activity, meaning that it can be safely administered with little to no adverse and life-threatening effects (Buijsers, *et al*., 2020). A large proportion of the antiviral research directed at heparin repurposing is related to respiratory infections, meaning that heparin would need to be

administered through inhalation. Data has shown that the pulmonary delivery of heparin is primarily mediated in the upper lung, meaning that it is not confined to particles of a certain geometric or aerodynamic diameter (Lewis, 2006). Furthermore, blood levels of heparin or LMWH were comparable to that of subcutaneous administration, however, the onset if action is much faster (Lewis, 2006). Unfractionated heparin is evidenced to have a positive effect in different acute lung injury models, specifically on pulmonary coagulation, inflammation and oxygenation (Chimenti *et al*., 2017). Smaller human studies demonstrate that nebulised UFH limits pulmonary fibrin deposition and attenuates the progression of acute lung injury, decreasing time to recovery (Camprubí-Rimblas *et al*., 2018) (Tuinman *et al*., 2012). A double-blind randomised study revealed that nebulised UFH limited progression of lung injury including acute respiratory distress syndrome and similarly hastened recovery (Dixon *et al*., 2021). Clinical study of the effect of inhaled nebulised unfractionated heparin on lung function in moderate to very severe COPD has shown that lung function improves over 21 days of treatment and significantly improved exercise capacity and dyspnoea with no adverse effects on coagulation (Dixon *et al*., 2021). Despite several studies showing that heparin mitigates both the onset and progression of lung injury, other large scale meta-analyses demonstrate no convincing evidence for the benefit of heparin nebulisation in intubated and ventilated ICU patients (Glas *et al*., 2016). Although the outlook is promising given the successful clinical data of nebulised heparin in a number of different lung pathologies, there is a need for more high quality randomised controlled trials of a larger size with long term follow up to ensure the safety and efficacy of nebulised heparin long term.

3. Coronaviruses

3.1. SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel coronavirus was identified as the causative agent for a cluster of pneumonia cases initially detected in Wuhan, China (Ghinai, *et al*., 2020). Severe acute respiratory syndrome coronavirus has been classified as a betacoronavirus of group 2B and is the cause of a serious life-threatening disease of 2019 (SARS-COV-2) (Li, *et al*., 2020). SARS-CoV-2 has been found to infect more people than either of its predecessors, SARS-CoV and the Middle East respiratory syndrome (MERS-COV) virus (Guarner, 2020). The origins of the virus are as yet undetermined, with speculation that the animal markets of Wuhan are responsible for the first human transmission of SARS-CoV-2 in late December of 2019 (Zhou, *et al*., 2020). The first case was reported by the WHO on December 31st, 2019, however, it is thought that the earliest case of SARS-COV-2 was detected as early as November 17th (Allam, 2020). The public health impact of the SARS-CoV-2 pandemic has been huge, it has affected more than 210 countries, the majority of which are still under some infection control measures (European Centre for Disease Prevention and Control, 2021). Following the primary report of SARS-COV-2,

reports of spread began to occur globally, reaching pandemic proportions. On the 30th of January 2020, the WHO declared the outbreak a Public Health Emergency of International concern, following this, on the 11th of March, the outbreak was declared a global pandemic (World Health Organisation, 2020). Latest reports, (as of 2nd of August 2021), show that there have been over 198,000,000 total cases globally and more than 4,000,000 global deaths attributed to SARS-CoV-2 (World Health Organisation, 2021). These numbers are a conservative value as retrospective assessment for many of these regions such as Africa, South Asia and the Western Pacific regions has demonstrated that there was a number of cases and deaths that were not recorded (Tovani-Palone *et al.,* 2022).

In January 2020, there was strong clinical evidence confirming the human transmission of SARS-CoV-2 (Mackenzie & Smith, 2020). Transmission of SARS-CoV-2 is primarily via respiratory droplets as a result of face-to-face contact and to a lesser degree through contaminated surfaces (Bourouiba, 2020) (Reynolds, *et al*., 2016). Aerosol spread may occur, yet the role of this has not yet been fully elucidated due to the lack of recoverable viral culture samples of SARS-CoV-2 (Heneghan, *et al*., 2021). Given that there is often gastrointestinal involvement, and SARS-CoV-2 has been detected in faeces, it is possible that faecal-oral transmission can occur (Guo, *et al*., 2021). For transmission to occur, symptoms do not have to be present, with an estimated 30-57% of transmission thought to occur in the presymptomatic stage (He, *et al*., 2020). Early in infection, SARS-CoV-2 targets cells of the upper respiratory tract, including nasal and bronchial epithelial cells and pneumocytes (Wiersinga, *et al*., 2020) (Gallo, *et al*., 2020) via the viral structural spike (S) protein that binds to the angiotensin-converting enzyme 2 (ACE2) receptor (Zamorano Cuervo & Grandvaux, 2020). The type 2 transmembrane serine protease (TMPRSS2), present in host cells promote viral uptake by cleaving ACE2 to activate the SARS-CoV-2 spike protein which mediates viral cell entry (Hoffmann, *et al*., 2020). In later stages of infection, when viral replication accelerates, epithelial-endothelial barrier integrity becomes compromised, SARS-CoV-2 infects

pulmonary capillary endothelial cells, inducing an inflammatory response and triggering an influx of monocytes and neutrophils (Chang, *et al*., 2021). Furthermore, diffuse thickening of the alveolar wall with mononuclear cells and infiltration of airspaces by macrophages has been reported post-mortem (Carsana, *et al*., 2020).

The incubation period of SARS-CoV-2 is generally between 3 and 7 days, with the shortest being 1 day and most within 2 weeks (Dhouib, *et al*., 2021). It is thought 80% of infections are mild to moderate, including pneumonia and non-pneumonia cases, with 13.1% developing severe disease and a further 6.1% develop critically serious disease requiring hospital intervention (World Health Organisation, 2020). Typical presentation of symptomatic SARS-COV-2, include fever (70-90%), dry cough (60-86%), fatigue (38%), shortness of breath (53-80%), weakness (25%), myalgias (15-44%), nausea and vomiting or diarrhoea (15-39%), rhinorrhoea (7%), and ageusia and anosmia (3%) (Docherty, *et al*., 2020) (Wiersinga, *et al*., 2020). Severe SARS-COV-2 symptoms usually occur within approximately 7 and 14 days after the onset of symptoms (Feng, *et al*., 2020). Clinically severe SARS-COV-2 has been defined as presence of tachypea, oxygen saturation ≤93% at rest, >50% lung involvement on imaging or PaO2/FiO2 ratio <300mmHg, whilst critical disease has been defined as respiratory failure requiring mechanical ventilation, septic shock or other organ dysfunction or failure requiring intensive care support (Attaway, *et al*., 2021). The risk of severe disease is highest in the elderly (>60 years) and those with comorbidities like diabetes, hypertension, obesity, chronic respiratory disease, cardiovascular disease, liver disease and malignancy (Sanyaolu, *et al*., 2020).

Mild to moderate cases of SARS-COV-2 can be managed easily with symptomatic therapy such as rest and over the counter medication (World health Organisation, 2020). Critical patients require admission to hospital and their management involves supportive care and management of complications AKI, ARDS, arrhythmias, cardiomyopathy, gastrointestinal bleeding, secondary bacterial infection, thromboembolism and polyneuropathy and myopathy (Adil, *et al*., 2021). Meta-analyses and systematic reviews have revealed that as many as 12% of patients with SARS-CoV-2 had co-infections (defined as recovery of other respiratory pathogens in patients with SARS-CoV-2 infection at time of diagnosis) and as many as 14% of patients had superinfections (defined as the subsequent recovery of other respiratory pathogens during care for patients infected with SARS-CoV-2), the latter is associated with poor outcomes *(Musuuza et al., 2021)*.In SARS-CoV-2, as primarily a respiratory pathogen, most patients who deteriorate often require respiratory support like assisted ventilation, with endotracheal intubation (World Health Organisation, 2020). One of the hallmarks of this pandemic, is the overwhelming number of critical care admissions on a daily basis in the early stages, which often exceeded the hospital capacity, evidenced in Italy and the UK and USA (Armocida, *et al*., 2020) (McCabe, *et al*., 2020) (Maves, *et al*., 2020).

In the UK of the available oral compounds with broad spectrum antiviral activity, only ribavirin is licensed to treat multiple virus strains, and this is typically in combination with other drugs (De Clercq & Li, 2016). However, due to its toxic effects is unlikely to be suitable as a treatment for SARS-CoV-2 and has subsequently been found to have no effect on negative conversion time or mortality rate (Tong, *et al*., 2020). Heparin has recently been approved by the World Health Organisation as an approved prophylactic treatment for patients hospitalised with SARS-COV-2 to prevent venous thromboembolism (World health Organisation, 2020). Further options include favipiravir and nitazoxanide, which have been shown to inhibit a large number of viruses *in vitro*, however, phase III trials have only been completed for influenza (Wang, *et al*., 2020). Favipiravir has recently become a part of 'PRINCIPLE', one of the UK governments national priority platform trials for SARS-COV-2 treatments for at home recovery (UK Research and Innovation, 2021). It has been found that nitazoxanide does not aid symptom resolution after 5 days of therapy but early nitazoxanide treatment reduced viral load significantly, with no observable adverse effects (Rocco, *et al*., 2020). Medications against other pathogens may also have anti-SARS-CoV-2 activity, yet many repurposing attempts have not been successful, including azithromycin (PRINCIPLE Trial Collaborative Group, 2021), hydroxychloroquine (Omrani, *et al*., 2020) and ivermectin (Popp, *et al*., 2021). Severe acute respiratory syndrome coronavirus 2 has a very short replication time, generating a large number of copies, increasing the likelihood of mutations that could confer resistance against antiviral drugs (Harvey, *et al*., 2021). This means that combination therapy may be required in order to provide effective treatment (Scientific Advisory Group for Emergencies, 2021). SARS-CoV-2 does present a few potential targets for antiviral therapy, the most significant of which is the ACE2 receptor, TMPRSS2 (Hu, *et al*., 2021). An inhibitor of this receptor, camostat, appears safe in humans and is currently being investigated in phase II trials (ClinicalTrials, 2020). Other targets include the vesicle transport system, such as the enzyme PI-3P-5-kinase, which is used to enter the host cell membrane (Beziau, *et al*., 2020). *In vitro* studies of an inhibitor apilimod look promising, however there is concern regarding the aggravation of immunosuppression in many SARS-COV-2 patients (Baranov, *et al*., 2021).

In October 2021, interim results of a clinical trial found that of 762 patients the number who needed to be admitted to hospital or who died was halved among those taking molnupiravir when compared with placebo (Jayk Bernal *et al*., 2022). This early data identified molnupiravir as a potential oral treatment for SARS-CoV-2, differing from the monoclonal antibody tocilizumab or the antiviral remdesivir which must be administered by intravenous infusion in hospitals (Gupta and Leaf, 2021) (WHO Solidarity Trial Consortium, 2022). Both molnupiravir and remdesivir impact the function of RdRp, where remedesivir shuts down RdRP function altogether, molnupiravir increases the frequency of viral RNA mutations and impairs SARS-CoV-2 replication in animals as RdRp utilises the active form of molnupiravir as a substrate instead of cytidine triphosphate or uridine

triphosphate (Menéndez-Arias, 2021). However, following this the full set of data released in December 2021, revealed that hospital admissions were only approximately 30% lower in the molnupiravir group (Jayk Bernal *et al*., 2022). In November 2021, the UK Medicines and Healthcare Products Regulatory Agency became the first to authorise molnupiravir through a conditional marketing authorisation (Medicines and Healthcare products Regulatory Agency, 2021). Following this, in December 2021, the US Food and Drug Administration also granted molnupiravir early use authorisation, only for use for the treatment of mild to moderate SARS-CoV-2 in at risk adults where other treatment options are not accessible or appropriate (The US Food and Drug Administration, 2021). Shortly after Japan granted special approval for molnupiravir and South Korea issued emergency approval in March 2022 (Pharmaceutical Evaluation Division, Pharmaceutical Safety and Environmental Health Bureau Ministry of Health, Labour and Welfare, 2021) (Yoo, 2021). France cancelled its order of 50,000 doses of molnupiravir in October 2021, due to efficacy concerns and the Indian Council of Medical research excluded molupiravir from its SARS-CoV-2 treatment guidelines over toxicity concerns (European Medicines Agency, 2021) (Kaur, 2022). In March 2022, WHO stated that molnupiravir should be provided only in cases of non-severe SARS-CoV-2 disease with higher risk of hospital admission (World Health Organization, 2022). Specifically, this means older people, those who are unvaccinated, have immunodeficiencies or chronic diseases. Populations excluded from treatment include children and pregnant and breastfeeding people.

As a result of the lack of approved treatments for SARS-CoV-2, attention turned to vaccine generation (ElBagoury, *et al*., 2021). In the UK there are currently three vaccines approved for use, two of which are mRNA vaccines: Moderna and Pfizer and AstraZeneca an adenovirus vaccine (Medicines and Healthcare products Regulatory Agency, 2021). Moderna and Pfizer have developed mRNA-based vaccines that have 85-95% efficacy against symptomatic SARS-CoV-2 and reduces hospitalisation by 90-99% (data relates to when the Alpha variant dominated) (Public Health England, 2021). Viral vector vaccines, as produced by AstraZeneca, use replication-deficient viruses engineered to express the genetic sequence of the antigen of interest in host cells using adenovirus vectors (Krammer, 2020). The AstraZeneca vaccine has an efficacy of 70-85% against symptomatic disease and decreases hospital admission by 70-85% (data relates to when the Alpha variant dominated) (Public Health England, 2021). However, on the 7th of April 2021, following benefit and risk analysis by the Joint Committee on Vaccination and Immunisation, advised that for adults aged under 30 years of age without underlying health conditions, should be given an alternative to AstraZeneca if available (Joint Committee on Vaccination and Immunisation, 2021). In early 2021, concerns were raised about vaccine escape, due to the rapid speed at which SARS-CoV-2 mutates, the efficacy of current vaccines has been under question (Andreano & Rappuoli, 2021). Globally, as of May 2022, only 57 countries have vaccinated 70%

of their population, most of which are high-income countries (World Health Organisation, 2022a). More than 30 vaccines have been approved for general or emergency use in countries around the world. Outside of the UK and US, other leading vaccines with emergency or limited approval in at least one country include inactivated vaccines by Sinopharm, CoronaVac, Covaxin, viral vector Sputnik V, Convidecia, Janssen and protein subunit Corbevax and Novavax (World Health Organisation, 2022b). The relative high infectivity, upper respiratory mode of transmission, relatively long incubation period, and the long viral shedding period, together global travel patterns, all contribute to the quick evolution of SARS-CoV-2 (Wang, *et al*., 2021). It has been revealed that a number of SARS-CoV-2 mutations, such as N439K, S477N, S477R and N501T strengthen the binding between the receptor binding domain and ACE2 (Wang, *et al*., 2021), indicating the virus is evolving to become more infectious. Further, the mutation N501Y, present in the UK, South Africa and Brazil variants may weaken the binding affinity between the receptor binding domain and many known antibodies (Wang, *et al*., 2021). Genetic evolution of SARS-CoV-2 on the receptor binding domain, may give rise to more infectious variants that may act to compromise existing vaccines and antibody therapies (Lauring & Hodcroft, 2021).

Due to the potential for vaccine escape and the lack of success thus far with the approval of SARS-CoV-2 treatments, it seems that drug repurposing should remain the focus of investigation. The advantages of which include, reduced risk during clinical trials compared to new treatments, as many adverse effects are already known, more cost effective as time to clinical trial is often shorter than novel drugs and can be undertaken in less time- so can be rapidly deployed (Singh, *et al*., 2020). Heparin appears to be a favourable compound for repurposing, as it has displayed a broad range of antiviral properties, exerts anti-inflammatory effects and has already been approved for treatment of complications in hospitalised patients.

3.2. Coronavirus Family:

Coronaviruses (CoVs) are highly diverse and constitute the largest group of viruses belonging to the *Nidovirales* order, which is comprised of the *Arteriviridae, Coronaviridae, Mesonviridae* and *Roniviridae* families (Woo, *et al*., 2012). The *Coronavirdae* comprise one of two families in the *Coronavirineae* family, with the other being *Torovirinae (Payne, 2017). The Coronaviridae* are further divided into four genera, alpha, beta, gamma and delta coronaviruses, initially this division was based on serology, however they are currently divided by phylogenetic clustering (Fehr & Perlman, 2015). Alphacoronaviruses and betacoronaviruses exclusively infect mammalian species, whereas gammacoronaviruses and deltacoronaviruses infect a wider range of hosts, including avian

species (V'kovski, *et al*., 2020). Human and animal coronavirus infections typically result in respiratory and enteric diseases (Domanska-Bilcharz, *et al*., 2020).

All viruses in the *Nidovirales* order are enveloped, non-segmented positive-sense strand RNA viruses (+ssRNA), approximately 120-160nm in diameter (Pal, *et al*., 2020). Atypically for RNA viruses, coronaviruses contain very large genomes, with some viruses in the family having the largest identified genomes, containing up to 35.5kb (Lai & Cavanagh, 1997). Genome size is significant as coronaviruses have the largest RNA genomes and are the only RNA virus family for which a 3'-exonuclease proofreading activity has been demonstrated (Ulferts and Ziebuhr, 2011), in addition there is a weak but significant negative association between genome size and rate of molecular evolution among RNA viruses (Sanjuán, 2012). However, as demonstrated in, *figure 8,* and ongoing research shows that SARS-CoV-2 has a number of mutations, with more potent variants emerging that the vaccines are not as effective against (Konishi, 2022). Other common features of this order include, highly conserved genomic organisation, with a large replicase gene preceding structural and accessory genes, expression of many non-structural genes by ribosomal frame-shifting, several unique or unusual enzymatic activities encoded within the large replicase-transcriptase polyprotein and expression of downstream genes by 3' nested sub-genomic mRNAs (Wang, *et al*., 2020).
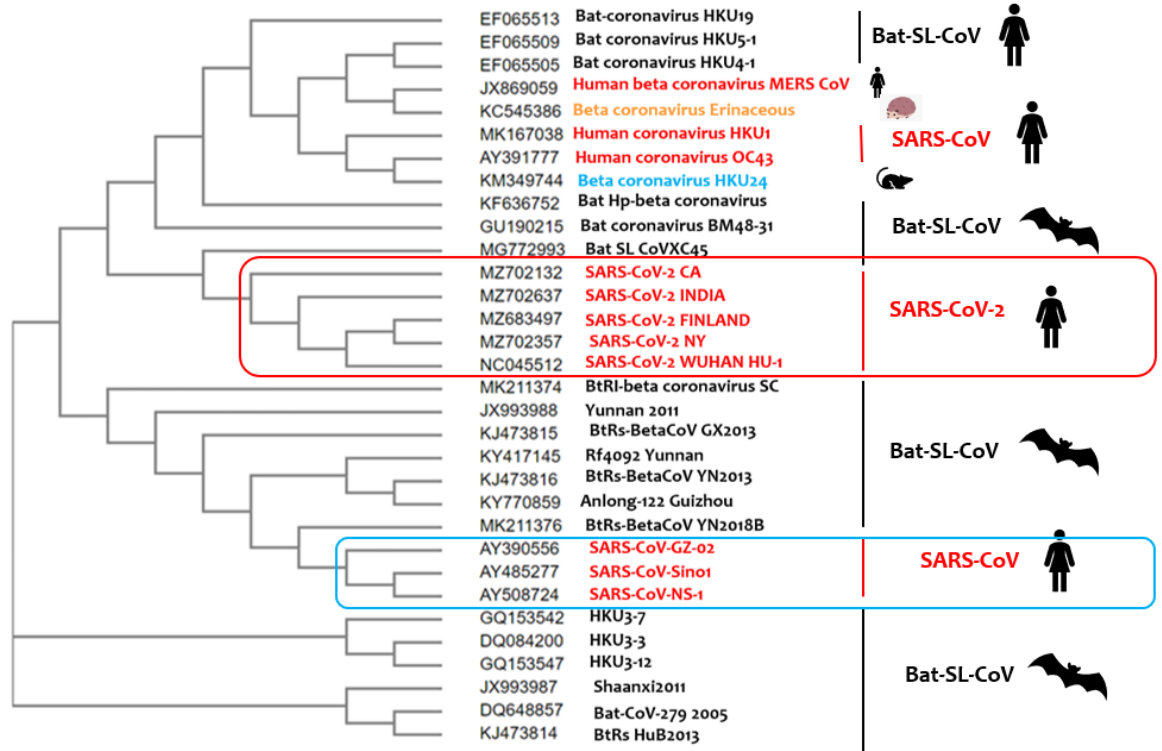
Figure 8: Phylogenetic analysis of full-length genomes of 2019-nCoV and representative viruses of the genus Betacoronavirus. Produced using most recent data at time of completion, 6th of August 2021. 32 coronavirus DNA sequences were analysed by pairwise and multiple alignment of 32 coronavirus DNA sequences via ClustalW, to produce a phylogenetic tree representing the evolution of each virus strain. As shown, red box, the novel SARS-CoV-2 is in a different clade to other SARS-CoV viruses and appears to be more closely related to BtRs-BetaCoV YN2018. MERS-CoV: Middle East respiratory syndrome coronavirus. SARS-CoV:severe acute respiratory syndrome coronavirus.

Coronaviruses such as HCoV-229-E and HCoV-OC43, HCoV-NL63 and HCoV-HKU1 have been known to circulate in the population to cause seasonal and often mild respiratory tract infections, similar in presentation to the common cold (Abdul-Rasool & Fielding, 2010). However, highly pathogenic variants such as, SARS-MERS-COV, SARS-CoV and SARS-CoV-2 have emerged in the human population over the past 20 years (Song, *et al*., 2019). These viruses infect bronchial epithelial cells, pneumocytes and upper respiratory tracts in humans to cause infection that can develop into severe and life-threatening respiratory pathologies and lung damage (V'kovski, *et al*., 2020). Currently there are no specific prophylactic or therapeutic treatments approved for these strains (Carvalho Nascimento, *et al*., 2020).

4. Coronavirus structure

4.1. Genomic organisation

Coronaviruses contain a non-segmented positive sense RNA genome of approximately 26-32kb (Denison, *et al*., 2011). The genome contains a 5' cap structure along with a 3'poly-adenine tail allowing it to act as an mRNA for translation of replicase polyproteins (Denison, *et al*., 2011). The size of the genome is among the largest known viral genomic RNAs, approximately three times the size of alphavirus or flavivirus genomes (Masters, 2006).
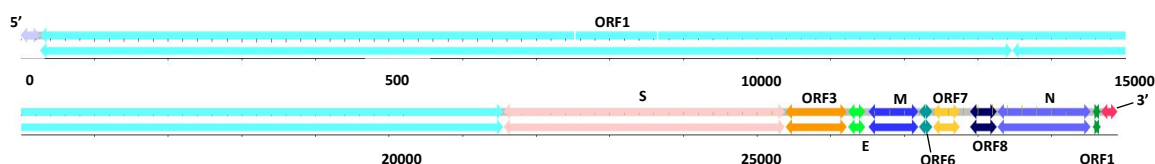
Figure 9: Coronavirus genome organisation. *The severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, GenBank: MN908947* (Wu, et al., 2020)*, complete genome was plotted using DNAplotter.*

This genomic RNA has three roles during the viral lifecycle: to act as the initial RNA of the infectious cycle, to act as a template for replication and transcription and finally, as a substrate for packaging into the progeny virus (Lim, *et al*., 2016). Approximately two thirds, ~20kb, of the 5' end of the genome is occupied by two large overlapping reading frames (ORFs), ORF1a and 1b (Cong, *et al*., 2017). The remaining 10kb at the 3' end is comprised of the structural and accessory protein genes which are transcribed into a nested set of subgenomic RNAs containing ORFs for the structural proteins (Cong, *et al*., 2017). The 5' end of the genome contains a leader sequence and untranslated region (UTR) that contains multiple stem loop structures necessary for RNA replication and transcription (Raman & Brian, 2005). In addition, transcriptional regulatory sequences (TSRs) precede each structural and accessory proteins, these are required for viral RNA replication and synthesis (Sola, *et al*., 2015). The 3' UTR also contains RNA structures required for replication and synthesis of viral RNA (Yang & Leibowitz, 2015). The organisation of the coronavirus genome is '5'-leader-UTR-replicase-S(spike)-E(Envelope)-M(Membrane)-N(nucleocapsid)-3'UTR-poly-A tail', this is a highly conserved gene order, with accessory genes interspersed within the structural genes at the 3' end of the genome (Pal, *et al*., 2020). The number and location of accessory proteins vary among CoV species and are almost exclusively non-

essential for replication in tissue culture, despite this, some have been shown to have important roles in viral pathogenesis (Fehr & Perlman, 2015) (Zhao, *et al*., 2010).

4.2. Virion structure

Coronavirus virions are roughly spherical and moderately pleiomorphic with diameters of ~125nm, as shown in cryo-electron tomography and cryo-electron microscopy (Kolesnikova, *et al*., 2003). Their most prominent and defining feature is the club-shaped spike projections emanating ~17-20nm from the virion surface (Petrov, 2020). Within the envelope of the virion is the nucleocapsid that encloses the ss+RNA of approximately 26-32kb (Denison, *et al*., 2011). Coronaviruses are unusual in that unlike most positive sense-RNA viruses they have helically symmetrical nucleocapsids, which is a more common feature in negative-sense viruses (Barcena, *et al*., 2009).

Coronavirus particles are comprised of four main structural proteins, which are the spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins all of which are encoded in the 3' end of the viral genome (Mousavizadeh & Ghasemi, 2020). The S protein (~150kDa) utilises an N-terminal signal sequence to gain access to the endoplasmic reticulum and is heavily N-glycosylated, increasing the apparent molecular weight by ~40kDa (Wang, *et al*., 2020). HomotriMERS-CoV of the virus encoded S protein make up the distinctive spike structure on the surface of the virus (Fehr & Perlman, 2015). The trimeric S glycoprotein is a class I fusion protein and binds to the host cell receptor to mediate the earliest steps of infection. In most coronaviruses, S is cleaved by a host cell furin-like protease into two polypeptides, S1 and S2 (Belouzard, *et al*., 2012). The former, S1, forms the large receptor-binding domain of the S protein, whereas the latter forms the stalk of the molecule (Belouzard, *et al*., 2012).

The M protein is the most abundant structural protein in the virion, it is a small (~25-30kb) polytopic protein with three transmembrane domains thought to be responsible for the shape of the virion (Neuman, *et al*., 2011). It has a small N-terminal glycosylated ectodomain and a larger C-terminal ectodomain that is situated in the interior of the virion or on the cytoplasmic face of intracellular membranes extending 6-8nm into the viral particle (Nal, *et al*., 2005). M protein is typically modified by N-linked glycosylation, however a subset of β-CoVs and δ-CoVs M proteins exhibit O-linked glycosylation (Oostra, *et al*., 2006). Despite being co-translationally inserted into the ER membrane, most M proteins do not contain a signal sequence (Locker, *et al*., 1992). Recent studies posit that the M protein exists in a dimerised form in the virion, adopting two conformations to allow both the promotion of membrane curvature and nucleocapsid binding (Neuman, *et al*., 2011). Recent studies suggest that the M protein of HCoV-NL63 has a role in the early stages of infection

by facilitating viral attachment to HSPGs which are utilised by HCoV-NL63 as initial attachment factors (Naskalaska, *et al*., 2019).

The E protein (~8-12kDa) is found in smaller quantities in the virion compared to M protein (Schoeman & Fielding, 2019). The coronavirus E proteins are highly divergent but share common structural features: a short hydrophilic N-terminal containing highly conserved cysteine residues followed by a hydrophobic region and a hydrophilic C terminal tail, containing conserved proline residues (Li, *et al*., 2014). The membrane topology of E protein is not completely elucidated; however, the majority of data suggests that it is a triple transmembrane protein (Schoeman & Fielding, 2019). The E protein has an N-terminal ectodomain and a C-terminal endodomain and has ion channel activity (Wang, *et al*., 2020). In contrast to other structural proteins, recombinant viruses without E protein are not always lethal, however, this is virus dependent (DeDiego, *et al*., 2007). The E protein has roles in assembly, budding and release of the virus, but has other functions too, for example, SARS-CoV E protein is not required for viral replication but is needed for viral pathogenesis (Ruch & Machamer, 2012). In addition to structural roles required to induce membrane curvature for viral assembly in coordination with viral M protein, E-protein mediates host immune responses via two distinct mechanisms: a pore forming transmembrane domain related to the activation of NLRP3 inflammasome (Nieto-Torres *et al*., 2015) and a PDZ (PSD-95/Dlg/ZO-1) binding function via its C terminal domain (Teoh *et al*., 2011) (Jimenez-Guardeño *et al*., 2014). Research has demonstrated that SARS-CoV-2 utilises this E protein PDZ binding function, to bind to the PDZ and SH3 domains of human cell junction protein PALS1 (Chai *et al*., 2021). This provides an explanation for the observed recruitment of PALS1 from lung epithelial cell junctions by viral E protein (Chai *et al*., 2021). Another immune evasion strategy utilised by coronaviruses is the use of glycans and other post-translational modifications to mask immunogenic viral protein epitopes. The envelope of SARS-CoV-2 is studded with glycoprotein spikes, comprised of homotrimers spike proteins heavily decorated with glycans (Zhao, Chen and Wang, 2021). Each spike protein is comprised of two subunits that each bear 22 glycan groups (Shajahan, Supekar, Gleinich and Azadi, 2020). In SARS-CoV-2, cell entry of the highly glycosylated S protein is enhanced by C-type lectin receptors, DC-SIGN, L-SIGN and the sialic acid-binding immunoglobulin-like lectin 1 (SIGLEC1) (Thépaut *et al*., 2021). Studies show that these C-type lectins function to augment ACE-2 mediated infection, modulating the neutralising activity of different classes of spike-spike antibodies, ultimately enhancing cell-to-cell fusion (Lempp *et al*., 2021).

The N-protein constitutes the only protein present in the helical nucleocapsid, it is comprised of two separate domains, an N-terminal (NTD) and a C-terminal domain (CTD), both of which are capable of *in vitro* RNA binding (Chen, *et al*., 2007). The mechanisms by which these occur differ for each terminal, it has been suggested that optimal RNA binding requires the involvement of both domains (Chen, *et al*., 2007). N protein is also heavily phosphorylated, this phosphorylation has

been suggested to trigger a structural change enhancing the affinity for viral versus nonviral RNA. N protein binds the viral genome in a 'beads on a string' type of conformation (Fehr & Perlman, 2015). N protein is known to interact with the viral M protein during virion assembly and has a vital role in improving the efficiency of virus transcription and assembly (McBride, *et al*., 2014). This is carried out by the binding of non-structural protein 3 (nsp3), a key component of the replicase complex and the M protein (McBride, *et al*., 2014). These proteins are thought to help tether the viral genome to the replicase-transcriptase complex (RTC) and subsequently package the encapsidated genome into viral particles (Fehr & Perlman, 2015). The RTC is thought to aid in the maintenance of the large genome of CoVs, it contains the unique 3'-5' exoribonuclease of nsp14 which is thought to provide a proofreading function to the RTC (Chen, *et al*., 2020).

Besides these four main structural proteins, a fifth structural protein, haemagglutinin-esterase (HE), is present in a subset of β-coronaviruses between ORF1b and the S gene (Lang, *et al*., 2020). The protein acts as a haemagglutinin, binding sialic acids on surface glycoproteins and contains acetyl-esterase activity (Lang, *et al*., 2020). This activity is thought to enhance S-protein mediated cell entry and virus spread through the mucosa by facilitating viral attachment to host cells (Zeng, *et al*., 2008).

5. Mechanisms of coronavirus infection and inhibition

5.1. Attachment and viral entry

The initial attachment of the virion to the host cell is initiated by interactions between the trimeric transmembrane S glycoprotein and its receptor (Belouzard, *et al*., 2012). The sites of receptor binding domains (RBD) within the S1 region of a coronavirus S protein vary dependent on the virus, with some having the RBD at the N-terminus of S1, for example murine β-coronavirus whilst other have the RBD at the C-terminus of S1 such as MERS-CoV (Qian, *et al*., 2015). The S-protein-receptor interaction is the primary determinant for coronavirus to infect a host species and also governs the tissue tropism of the virus (Qian, *et al*., 2015). Many coronaviruses utilise peptidases as their cellular receptor. The reason for this is unclear, as entry occurs even in the absence of the enzymatic domain of these proteins (Fehr & Perlman, 2015). Many α-coronaviruses utilise aminopeptidase N (APN) as their receptor (Delmas, *et al*., 1992), whereas β-coronaviruses have more varied receptors, for example BCoV utilises N-acetyl-9-O-acetylneuraminic acid whereas SARS-CoV utilises angiotensin converting enzyme 2 (ACE2) (see *Table 5*) (Li, 2015).

Table 5: Coronavirus Receptors. APN:*aminopeptidase N,* ACE2 *angiotensin-converting enzyme 2,* mCEACAM *murine carcinoembryonic antigen-related adhesion molecule 1,* DPP4 *dipeptidyl peptidase 4,* HCoV *human coronavirus,* TGEV *transmissible gastroenteritis virus,* PEDV *porcine epidemic diarrhoea virus,* FIPV *feline infectious peritonitis virus,* CCoV *canine coronavirus,* MHV *murine hepatitis virus,* BCoV *bovine coronavirus,* SARS-CoV *severe acute respiratory syndrome coronavirus,* MERS-COV-CoV *Middle East respiratory syndrome coronavirus*

Virus	Receptor	References
Alphacoronaviruses		
HCoV-NL63	ACE2	(Hofmann, et al., 2005)
TGEV	APN	(Delmas, et al., 1992)
PEDV	APN	(Li, et al., 2007)
FIPV	APN	(Tresnan, et al., 1996)
CCoV	APN	(Benbacer, et al., 1997)
Betacoronaviruses		
MHV	mCEACAM	(Williams, et al., 1991)
BCoV	*N*-acetyl-9-*O*-acetylneuraminic acid	(Schultze & Herrier, 1992)
SARS-CoV	ACE2	(Li, et al., 2003)

Following receptor binding, the fusion of the viral envelope to a host cell membrane occurs, mediated by viral transmembrane proteins, known as fusogens (Tang, *et al*., 2020). The virus subsequently gains access to the host cell, generally by acid-dependent proteolytic cleavage of S protein by a cathepsin, TMPRRS2 or another protease such as furin, trypsin and elastase (Zipeto, *et al*., 2020). The proteolytic cleavage of the S proteins is vital to induce the dissociation of S1 from S2, a trigger that is followed by the fusion of viral and cellular membranes (Zipeto, *et al*., 2020). In addition to receptor binding and cleavage of S proteins, pH dependent fusion can also occur (Belouzard, *et al*., 2012). S protein cleavage occurs at two sites within the S2 region of the protein, with the first cleavage important for separation of the RBD and fusion domains of the S protein and the second for exposing the fusion peptide (cleavage at S2') (Belouzard, *et al*., 2012). Fusion generally occurs within acidified endosomes, however, some coronaviruses, such as MHV, can fuse at the plasma membrane (Burkard, *et al*., 2014). Cleavage at S2' exposes a fusion peptide that inserts into the membrane, which is followed by the joining of two heptad repeats in the S2 to form an antiparallel six-helix bundle (Bosch, *et al*., 2003). The formation of this bundle allows for the mixing of viral and cellular membranes, resulting in fusion and ultimately release of the viral genome into the cytoplasm (Bosch, *et al*., 2003).

5.2. Replicase protein expression:

The proceeding step in the coronavirus lifecycle is the translation of the replicase gene from the virion genomic RNA. The replicase gene encodes two large open reading frames (ORFs), rep1a and rep1b, which express two co-terminal polyproteins, pp1a and pp1b (Wang, *et al*., 2020). To express both polyproteins, the virus utilises a slippery sequence (5'UUUAAAC-3') and a RNA pseudoknot structure that causes ribosomal frameshifting from the rep1a reading frame into the rep1b reading frame (Fehr & Perlman, 2015). Typically, the ribosome unwinds the pseudoknot structure, continuing translation until it encounters the rep1a stop codon (Bhatt, *et al*., 2020). However, on occasion, the pseudoknot blocks the ribosome from continuing elongation, causing it to pause on the slippery sequence (Fehr & Perlman, 2015). This alters the reading frame by moving back one nucleotide to cause a -1 frameshift, before the ribosome is able to melt the pseudoknot structure and extend translation into rep1b, leading to pp1ab translation (Fehr & Perlman, 2015). *In vitro* studies have predicted the incidence of ribosomal frameshifting to be as high as 25%, however, this has not been determined in the context of viral infection (Bhatt, *et al*., 2020). The purpose of frameshifting to control protein expression has not yet been elucidated, but it is thought that it is to either control the ratio of rep1b and rep1a proteins or delay the production of rep1b products until the products of rep1a have created a suitable environment for RNA replication (Plant & Dinman, 2008).

Subsequently, polyproteins pp1a and pp1ab are proteolytically cleaved by proteases encoded by the ORF1a, to contain the nsps 1-11 and 1-16 respectively (Fang, *et al*., 2008) (*see Table 6*). In pp1ab, nsp11 becomes nsp12 following extension of pp1a into pp1b (Fang, *et al*., 2008). However, γ-coronaviruses do not contain a comparable nsp1 (Fang, *et al*., 2008). These polyproteins are subsequently cleaved into the individual nsps (Fang, *et al*., 2008). Coronaviruses encode either two or three proteases, known as the papain-like proteases (PLpro), that cleave the replicase polyproteins (Baretto, *et al*., 2005). PLpro are encoded within nsp3 and a serine type protease known as the main protease (Mpro) is encoded by nsp5 (Baretto, *et al*., 2005). Most coronaviruses encode two PLpros within nsp3, except the γ-coronaviruses, SARS-Cov and MERS-CoV, which

only express one PLpro (Fehr & Perlman, 2015). The PLpros act to cleave the nsp1/2, nsp2/3 and nsp3/4 boundaries, whilst the Mpro is responsible for the 11 remaining cleavage events (Baez-Santos, *et al*., 2015).

Subsequently, many of the nsps assemble into the replicase-transcriptase complex (RTC) to form an environment suitable for RNA synthesis, ultimately promoting RNA replication and transcription of the sub-genomic RNAs (Wang, *et al*., 2020). The nsps have varying enzyme domains and functions including those important for RNA replication (Wang, *et al*., 2020). The main component of the RTC is Nsp12, which encodes the RNA dependent RNA polymerase (RdRp) domain which acts to directly mediate de novo primer-independent RNA synthesis during viral replication, it also mediates transcription of ORFs producing mRNAs for structural and accessory proteins (Zhu, *et al*., 2020). Additionally, nsp13 encodes the highly conserved helicase subunit necessary for efficient replication of the viral genome. Nsp14 encodes the bifunctional exoribonuclease (ExoN) involved in replication fidelity and N7-methyltransferase activity and nsp16 encodes 2'-O-methyltransferase activity (Romano, *et al*., 2020). In addition to the replication functions, other activities have been identified for some of the nsps, such as blocking innate immune responses (nsp1, nsp16-2'-O-methyl transferase, nsp3-deubiquitinase) whereas others have unknown functions (nsp3-ADP-ribose-1'-phosphatase, nsp15-endoribo-nuclease (NendoU)) (Romano, *et al*., 2020). The ribonucleases, nsp15 NendoU and nsp14-ExoN activities are unique to the Nidovirales order and are considered genetic markers for these viruses (Romano, *et al*., 2020).

Table 6: Functions of coronavirus non-structural proteins. PLPro: papain-like protease, Mpro: main protease, RdRp: RNA-dependent RNA polymerase, MTase: methyltransferase, ExoN: viral exoribonuclease, NendoU: viral endoribonuclease, 2'-O-MT: 2'-O-methyltransferase, MDA5: *melanoma differentiation associated protein.*

Protein	Function	Reference
nsp1	Promotes cellular mRNA degration and blocks host cell translation. Results in blocking innate immune response by inhibiting type I IFN.	(Huang, et al., 2010) (Kamitani, et al., 2009) (Kamitani, et al., 2006) (Tanaka, et al., 2012)
nsp2	No known function, binds prohibitin proteins	(Cornillez-Ty, et al., 2009) (Graham, et al., 2005)
nsp3	PLPro, polypeptides cleaving, blocking host innate immune response, promoting cytokine expression	(Ziebhur, et al., 2001) (Egloff, et al., 2006) (Serrano, et al., 2007) (Neuman, et al., 2008) (Eriksson, et al., 2008) (Chatterjee, et al., 2009) (Frieman, et al., 2009) (Serrano, et al., 2009)
nsp4	Potential transmembrane scaffold protein, important for double membrane vesicle formation	(Clementz, et al., 2008) (Gadlage, et al., 2009)
nsp5	Mpro cleaves viral polyprotein, inhibits type I IFN signalling	(Lu, et al., 1995)
nsp6	Potential transmembrane scaffold protein, important for proper structures of double membrane vesicles	(Oostra, et al., 2008)
nsp7	Forms hexadecasaccharide complex with nsp8, may act as processivity clamp for RNA polymerase	(Zhai, et al., 2005)
nsp8	Forms hexadecasaccharide complex with nsp7, may act as processivity clamp for RNA polymerase, may act as primase	(Zhai, et al., 2005) (Imbert, et al., 2006)
nsp9	RNA binding protein	(Egloff, et al., 2004)
nsp10	nsp16 and nsp14 cofactor (forms heterodimer with both), stimulates ExoN and 2-O-MT activity	(Bouvet, et al., 2010) (Decroly, et al., 2011)
nsp12	RdRp	(Xu, et al., 2003)
nsp13	RNA helicase, 5' triphosphatase	(Ivanov, et al., 2004) (Ivanov & Ziebuhr, 2004)
nsp14	N7 MTase and 3'-5' exoribonuclease, ExoN; N7 MTase adds 5' cap to viral RNAs, ExoN activity is important for proofreading of viral genome	(Minskaia, et al., 2006) (Erckerle, et al., 2007) (Chen, et al., 2009) (Erckle, et al., 2010)
nsp15	Viral endoribonuclease, NendoU	(Ivanov, et al., 2004) (Bhardwaj, et al., 2006)
nsp16	2'-O-MT; shields viral RNA from MDA5 recognition	(Decroly, et al., 2008) (Zust, et al., 2011)

5.3. Assembly and release:

Viral RNA synthesis follows the translation and assembly of the viral replicase complexes. Viral RNA synthesis produces both genomic and sub-genomic RNAs from the RTC via negative-strand intermediates (Sola, *et al.*, 2015). The latter serve as mRNAs for the structural and accessory genes, translated into the membrane bound S, M, and E proteins to form a set of nested RNAs distinctive of the order *Nidovirales* and the accessory proteins (Sola, *et al.*, 2015).

After replication and sub-genomic RNA synthesis, the viral structural proteins, S, E and M are translated and inserted into the endoplasmic reticulum (ER), where they move along the secretory pathway into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (Ujike &

Taguchi, 2015). There, viral genomes encapsidated by N-protein bud into membranes of the ERGIC containing viral structural proteins, forming mature virions (Ujike & Taguchi, 2015). Following virion assembly, transportation of progeny virions away from infected cells occurs in vesicles to the plasma membrane, virions are then released by exocytosis (de Haan & Rottier, 2005). It not known whether virions use the traditional pathway for transport of large cargo form the Golgi or if the virus has a diverted, unique pathway for its own exit (Wang, *et al*., 2020). In several coronaviruses, S protein that does not get assembled into virions transits to cell surface where it mediates cell-cell fusion between infected cells and adjacent, uninfected cells (Schoeman & Fielding, 2019). During infection of some coronaviruses, but not all, S protein that has not been assembled into virions reaches the plasma membrane (Schoeman & Fielding, 2019). At the cell surface S protein can cause the fusion of infected cells and adjacent uninfected cells (Wang, *et al*., 2020). This subsequently leads to the formation of giant, multinucleated cells, allowing viral spread within an infected organism evading detection or neutralisation by viral specific antibodies (Wang, *et al*., 2020).

6. Diversity of coronavirus pathogenesis:

Coronaviruses display diverse host range and tissue tropism, typically α-coronaviruses and β-coronaviruses infect mammals, whereas γ-coronaviruses and δ-coronaviruses infect birds and fish, however some have been known to infect mammals (Guo, *et al*., 2020). Prior to 2019, there were only six CoVs known to infect humans and cause respiratory disease: HCoV-229E, HCoV-OC43, HCoV-NL63, and HKU1, SARS-CoV and MERS-COV-CoV (Liu, *et al*., 2020). The first five, HCoV-229E- HCoV-HKU1, cause mild upper respiratory disease in immunocompetent hosts and in rarer cases cause a severe infection in infants, young children, and elders (Liu, *et al*., 2020). Whereas SARS-CoV and MERS-CoV and the novel SARS-CoV-2 can infect the both the upper

and lower respiratory tract and cause severe respiratory syndrome in humans (Zhu, *et al*., 2020). Furthermore, extra-pulmonary involvement is reported in the hematologic, cardiovascular, renal, gastrointestinal, and hepatobiliary, endocrinologic, neurologic, ophthalmologic, and dermatologic systems (Montero, 2021). This pathology reflects either extrapulmonary dissemination and replication of SARS-CoV-2 as has been observed in other zoonotic coronaviruses, or widespread immunological and pathological changes and sequelae of the disease (Yao, Lu and Ma, 2022). On the basis of current sequence databases all human coronaviruses have animal origins: SARS-CoV-2, SARS-CoV, MERS-CoV, HCoV-NL63 and HCoV-229E are considered to have originated in bats, whereas HCoV-OC43 and HKU1 are likely to have originated from rodents (Hu, *et al*., 2017). Domestic animals may have important roles as intermediate hosts that enable the transmission from natural hosts to humans (Ye, *et al*., 2020).

Prior to the emergence of SARS-CoV, there were two prototype human coronaviruses, OC43 (β-coronavirus) and 229E (α-coronavirus)- both aetiological agents of the common cold (Varet, *et al*., 2003). There had been speculation regarding the association of human coronaviruses with more serious human pathologies such as enteric disease in new-borns (Resta, *et al*., 1985), hepatitis, and multiple sclerosis (Burks, *et al*., 1980), however, there is no scientific evidence as of yet to substantiate these claims. The first HCoV-229E strain was isolated in 1966, from the respiratory tract of patients with upper respiratory tract infections, this sample was adapted to grow in WI-38 cell lines (McIntosh, *et al*., 1967). Patients presented with symptoms similar to the common cold, acute rhinorrhoea, nasal congestion, sore throat, malaise, headache chills and cough (McIntosh, *et al*., 1967). The following year, 1967, HCoV-OC43 was isolated from tracheal and nasal organ culture and initially propagated on ciliated human embryonic tissue, before serial passage in brains of suckling mice (McIntosh, *et al*., 1967b). Clinically the symptoms of HCoV-OC43 are very similar to those caused by HCoV-229E, which are indistinguishable from other common respiratory tract infections caused by influenza A viruses and rhinoviruses (Vabret, *et al*., 2003). Both HCoV-229E and HCoV-OC43 are distributed globally in temperate climates during winter, responsible for 10-30% of all common colds (Fields, *et al*., 1993).

SARS-CoV was the third HCoV discovered, the first case can be traced back to late 2002 in the Guangdong province of China, later resulting in 8098 reported cases causing 774 deaths with a case fatality rate of 9.7% globally (World Health Organisation, 2003). Aside from 'super-spreaders', each case was thought to give rise to approximately 2 secondary cases, with an incubation period of four to seven days and the peak viral load appearing on the 10th day of illness (Lam, *et al*., 2003). Patients typically present with chills, fever, malaise, myalgia and headaches later followed by cough, dyspnoea and respiratory distress (Hui, *et al*., 2004). Lymphopenia, deranged liver function tests and elevated creatine kinase are common laboratory abnormalities of SARS (Lee, *et al*., 2003), with diffuse alveolar damage, epithelial cell proliferation and macrophage increases also seen in

SARS-CoV infection of the lung (Gu & Korteweg, 2007). Approximately 20-30% of patients develop more severe disease, requiring intensive care and mechanical ventilation (Peiris, *et al*., 2003). The infection is not just isolated to the respiratory tract, with multiple organ involvement such as the gastrointestinal tract- with diarrhoea seen in approximately 30-40% of cases and the liver and kidneys in more severe cases (Shi, *et al*., 2005). These cases are usually accompanied by a cytokine storm, which can be lethal, with the most pronounced effects occurring in immunocompromised patients (Rock, *et al*., 2009). Following the isolation of the virus from an open lung biopsy in 2003, great efforts have been made to advance the field of HCoV research (Kaslow, *et al*., 2014).

HCoV-NL63 and HCoV-HKU1 were isolated in late 2004 and early 2005 respectively. HCoV-NL63 was primarily associated with younger children, the elderly and immunocompromised patients with respiratory illnesses (van der Hoek, *et al*., 2004). Infections with NL63 present as mild respiratory disease, similar to the common cold, typified by cough, fever, hypoxia, rhinorrhoea and tachypnoea that tends to resolve without intervention (Abdul-Rasool & Fielding, 2010). Obstructive laryngitis, croup, is often observed in HCoV-NL63 infection, with 43% of patients suffering from croup (van der Hoek, *et al*., 2005). It has been estimated that HCoV-NL63 accounts for 4.7% of common respiratory diseases globally, with peak incidence occurring during early summer, spring and winter (Su, *et al*., 2016). Similarly, HCoV-HKU1 is found globally and causes mild respiratory disease, however, it has been found to be associated with community-acquired bronchiolitis and pneumonia as well as acute asthmatic exacerbation (Ebihara, *et al*., 2005).

Prior to the novel SARS-CoV-2 strain, MERS-CoV was the most recent coronavirus identified. The first case was reported in Jordan in 2012, with the spread of this virus was mainly contained in the Middle East with occasional secondary spreads via imported cases in various European countries and Tunisia (Zaki, *et al*., 2012) (Mailles, *et al*., 2013). A second outbreak occurred in 2015 in South Korea, with 186 confirmed cases (World Health Organisation, 2015). Clinically patients present with symptoms similar to that of SARS-CoV, with a fever, cough, breathing difficulties and progressive acute pneumonia with diffuse alveolar damage (Das, *et al*., 2016). MERS-CoV has also been detected in more mild and influenza-like illnesses and asymptomatic patients (Mackay & Arden, 2015). However, unlike SARS, many patients with MERS-COV also develop acute renal failure, making MERS-CoV unique among the previously identified HCoVs (Chan, *et al*., 2015) and more rapidly progress to respiratory failure (Hui, *et al*., 2014) Similar to SARS-CoV, more than 30% of patients present with gastrointestinal symptoms such as diarrhoea and vomiting (Assiri, *et al*., 2013). As of February 14[th,] 2020, over 2500 laboratory confirmed cases were reported with a high case mortality of 34.4% (Park, *et al*., 2020)

6.1. Treatment and prevention:

Currently, there is no single specific antiviral therapy for CoVs, the main treatments are supportive, aiding in symptom relief as opposed to targeting the virus itself (Carvalho Nascimento, *et al*., 2020). Recombinant IFN with ribavirin has shown limited effects against CoVs infection (Falzarano, *et al*., 2013). To mitigate the risk of bacterial infections and complications prophylactic antibiotics are given (Bleibtreu, *et al*., 2018) (Zhou, *et al*., 2016). Following the epidemics of SARS-CoV and MERS-CoV, efforts have been made to develop new antivirals targeting CoVs proteases,

polymerases, MTases and entry proteins, however, none of which have proven to be effective in clinical trials (Carvalho Nascimento, *et al*., 2020).

The nucleoside analogue, ribavirin, shows antiviral activity against a variety of DNA and RNA viruses (Sidwell, *et al*., 1972). Ribavirin has been indicated for several DNA and RNA viruses, exerting antiviral activity via mutagenic effects in the treatment of poliomyelitis (Crotty, *et al*., 2000), respiratory syncytial virus (Herzog, *et al*., 1990), hepatitis C (Reddy, *et al*., 2009), haemorrhagic fever (Westover, *et al*., 2016) and influenza A and B (Stein, *et al*., 1987). Based on this research the empiric use of ribavirin and corticosteroids is widely used as a primary treatment of coronaviruses, despite uncertainty about its efficacy. This is exemplified by opposing studies, with regards to MERS-COV and ribavirin treatment. One retrospective cohort study investigating the use of ribavirin and interferon-alpha, showed a significant improvement in the 14-day survival rate of patients but not at 28 days (Omrani, *et al*., 2014). In contrast, a separate retrospective cohort study investigating ribavirin/interferon treatment in severe cases of MERS-COV suggested that this treatment did not significantly improve the rate of RNA clearance of MERS-CoV and was associated with higher 90-day mortality (Arabi, *et al*., 2019). In SARS, ribavirin and glucocorticoids were used as a first line treatment in critically ill patients, however, analysis of treatment outcomes has shown that there is little therapeutic benefit of these drugs in SARS patients (Lau, *et al*., 2009). Following the recent SAR-CoV-2 outbreak, an abundance of research has become available regarding the use of ribavirin as a first-line treatment. The use of ribavirin as a solo treatment showed that therapy in patients with severe SARS-COV-2 does not significantly improve negative conversion time for SARS-COV-2 test and is not associated with an improved mortality rate (Tong, *et al*., 2020). As ribavirin is traditionally given in combination with other drugs, there is a wealth of research investigating the efficacy as part of a cocktail. A triple-treatment of lopinavir-ritonavir, ribavirin and interferon beta-1b was assessed for efficacy and safety in patients with SARS-COV-2 (Hung, *et al*., 2020). The results of which were more promising, with the triple treatment found to improve symptoms, duration of viral shedding and hospital stay in patients with mild to moderate SARS-COV-2 when compared to lopinavir-ritonavir alone (Hung, *et al*., 2020). A similar study, but utilising an interferon-alpha backbone, as opposed to beta, investigated the efficacy of ribavirin and interferon-α, lopinavir-ritonavir and interferon-α and ribavirin, lopinavir-ritonavir and interferon-α (Huang, *et al*., 2020). Results indicated that there was no significant difference among the three regimens with regards to nucleic acid negativity, however, raw data showed that lopinavir-ritonavir with interferon-α was the most effect, but the difference was not statistically significant (Huang, *et al*., 2020). Results showed the triple therapy was associated with a significant increase in gastrointestinal adverse events, suggesting that ribavirin and lopinavir-ritonavir should not be administered in combination with SARS-COV-2 patients (Huang, *et al*., 2020).

Ribavirin displays obvious signs of toxicity and the use of higher doses of ribavirin in critically ill patients, as in MERS-CoV and SARS, can lead to progressive haemolytic anaemia, bradycardia, and hypomagnesemia (Muller, *et al*., 2007). The exact effects are unknown, due to the consistent combined use of ribavirin and other pharmaceuticals.

Remdesivir, a monophosphamidate prodrug of the C-adenosine analogue GS-441524 that inhibits viral RNA polymerases (Grein, *et al*., 2020), shows broad-spectrum, anti-coronavirus activity (Agostini, *et al*., 2018). With reports showing that it acts to inhibit the replication of SARS-CoV and MERS-CoV in human epithelial cells, improve lung function and reduce viral load (Sheahan, *et al*., 2017). Furthermore, remdesivir was found to be more effective than lopinavir-ritonavir against MERS-CoV *in vitro* (Sheahan, *et al*., 2020). Remdesivir was effective in controlling SARS-CoV-2 *in vitro* (Wang, *et al*., 2020), but more evidence is required to validate the role of Remdesivir in SARS-COV-2. A retrospective cohort study revealed that remdesivir was more effective than placebo in reducing the time to recovery in adults hospitalised with SARS-COV-2 with evidence of lower respiratory tract infection (Beigel, *et al*., 2020). Following this, the World Health Organisation released results from their large-scale SOLIDARITY trial tasked with researching the effect of four repurposed antiviral drugs in patients hospitalised with SARS-COV-2. This study revealed that regimens of all four drugs, including remdesivir, had little to no effect on hospitalised patients, with parameters that included overall mortality, initiation of ventilation and duration of hospital stay. (WHO Solidarity Trial Consortium, 2021). This research exposed further areas for research particularly to provide higher certainty of evidence for specific groups of patients, with a call to action for further clinical trials evaluating use, dosage, and indications (WHO Solidarity Trial Consortium, 2021).

Corticosteroids have been widely used during the previous outbreaks of SARS and MERS-COV; they were given empirically as the urgency of the outbreak did not allow time for efficacy studies. In the case of SARS, there are no studies evidencing the cytopathic effect of corticosteroids against SARS-CoV, due to the inability to measure direct antiviral effects as a result of their role as immunomodulatory agents (Stockman, *et al*., 2006). Results from clinical trials range from inconclusive to showing possible harm. A randomised double-blind placebo-controlled trial found that corticosteroid use in the first week of illness acted to delay viral clearance (Lee, *et al*., 2004). The adverse effects of corticosteroid treatment have been well evidenced, with links between psychosis and administration of steroids, as patients receiving higher doses of steroids and had higher rates of family history of psychiatric illness, had higher rates of SARS-related psychosis (Lee, *et al*., 2004). In addition, treatment with methylprednisolone has been associated with the onset of diabetes, with over-dose administration of methylprednisolone leading to a high frequency of diabetes (Xiao, *et al*., 2004). Another, uncontrolled retrospective study of 40 SARS patients, reported avascular necrosis and osteoporosis among corticosteroid-treated SARS patients (Li, *et*

al., 2004). Further research regarding the use of corticosteroid treatment in MERS-COV, revealed that there was no improvement in mortality after adjustment or time-varying confounders and acted to delay MERS-COV coronavirus clearance, as in SARS-CoV (Arabi, *et al*., 2017). The use of corticosteroids in SARS-CoV-2 has not been as widely documented, however, early data suggests that corticosteroids confer more risk than benefit in moderate cases (Cano, *et al*., 2021). This is consolidated by WHO guidelines that state corticosteroids should be utilised in low doses in only severe and critical manifestations of SARS-COV-2 (World Health Organisation, 2020). Despite these recommendations, further studies have revealed a protective role of corticosteroids at higher concentrations in selected cases of severe SARS-COV-2. The corticosteroid dexamethasone has been shown to have a beneficial effect on mortality in moderate and severe acute respiratory distress syndrome, with no excess adverse events when compared to the placebo (Villar, *et al*., 2020). Further research is required to elucidate the benefits of corticosteroid treatment, dosage requirements and patient need.

In vitro studies, preclinical studies and observational studies increased interest in the protease inhibitor lopinavir/ritonavir. Traditionally used in conjunction with other anti-HIV medicines, lopinavir acts as a HIV-1 protease inhibitor, combined with ritonavir to increase its plasma half-life (Chandwani & Shuter, 2008). Molecular dynamic simulations found that lopinavir and ritonavir also inhibit severe acute respiratory syndrome coronavirus (SARS-CoV) main protease, 3CL^{pro} [Nukoolkarn, *et al*., 2008]. *In vitro* studies reveal a less promising role for these drugs, with antiviral activity detected, but decreasing with incubation beyond 48 hours, with research indicating a supportive role of lopinavir/ritonavir when used with ribavirin (Chu, *et al*., 2004). Similarly, lopinavir/ritonavir was shown to have *in vitro* activity against MERS-COV, inhibiting MERS-CoV replication at low concentrations (EC$_{50}$= 3-8 μM) (de Wilde, *et al*., 2014). Further murine investigations demonstrated that the benefits of lopinavir/ritonavir are more significant when used prophylactically compared to therapeutically (Sheahan, *et al*., 2020). In contrast, lopinavir had similar anti-SARS-CoV-2 activity compared to remsedivir, with EC$_{50}$ values of 23.15 μM and 26.63 μM, respectively (Choy, *et al*., 2020). However, clinical trials have shown that these promising results do not translate when applied in person, with no benefit observed with lopinavir-ritonavir treatment when compared to standard care (Cao, *et al*., 2020). This finding was consolidated by the RECOVERY trial, which confirmed that there were no beneficial effects of lopinavir-ritonavir when compared to placebo (RECOVERY Collaborative Group, 2020). In addition to serving no clinical benefit, the use of lopinavir and ritonavir increases the likelihood of gastrointestinal events, including anorexia and nausea (Cao, *et al*., 2020) (Vecchio, *et al*., 2020)

7. SARS-CoV-2:

SARS-CoV-2 is an enveloped β-coronavirus with a genetic sequence similar to SARS-CoV-2 (80%) and animal coronavirus bat RaTG13 (96.2%) (Zhou, *et al*., 2020). Like other coronaviruses, the viral envelope is coated by spike glycoprotein, envelope, and membrane proteins (Cevik, *et al*., 2020). As in SARS-CoV-1, host cell binding is mediated by the S protein with the primary step of

infection being spike protein to target receptor binding (Shang, *et al*., 2020). However, the S gene of SARS-CoV-2 is highly variable from SARS-CoV, with 75% of nucleotide identity in common (Zhou, *et al*., 2020). With regards to SARS-CoV-2 the S1 subunit of the S protein contains the receptor binding domain that binds to the peptidase domain of angiotensin-converting enzyme 2 (ACE2) (Tai, *et al*., 2020). The S2 subunit it highly conserved and considered a potential antiviral target (Shah, *et al*., 2021). The S1/S2 polybasic cleavage site is proteolytically cleaved by cellular cathepsin L and the transmembrane protease serine 2 (TMPRSS2) (Jaimes, *et al*., 2020). This acts to facilitate viral cell entry at the plasma membrane surface (Hoffmann, *et al*., 2020), whereas cathepsin L activates SARS-CoV-2 Spike in endosomes and compensates for entry in cells lacking TMPRSS2 (Zhao, *et al*., 2021). Once the genome is released into the host cytosol, ORF1a and ORF1b are translated into viral replicase proteins, which are cleaved into individual non-structural proteins (via host and viral proteases: PL[pro]), these form the RNA-dependent RNA polymerase (nsp12 derived from ORF1a and ORF1b) (Harrison, *et al*., 2020). The replicase components rearrange the endoplasmic reticulum into double membrane vesicles (DMVs) that facilitate viral replication of genomic and sub-genomic RNAs (Mohan & Wollert, 2021). The latter are subsequently translated into accessory and viral structural proteins that facilitate virus particle formation (Harrison, *et al*., 2020).

SARS-CoV-2 is unique in its ability and prevalence of viral mutations (Islam, *et al*., 2020). Coronaviruses have the capacity for proofreading during replication, therefore, mutation rates are typically lower than in other RNA viruses (Robson, *et al*., 2020). The spread of SARS-CoV-2 has been global, as a result, mutations have been accumulated that contain geographical signatures (Bandoy & Weimer, 2021). To study virus characterisation and develop understanding of epidemiology and transmission patterns, these mutations have been examined (Cevik, *et al*., 2020). Overall, mutations have not been related to phenotypic changes affecting viral transmissibility or pathogenicity but associated with adaptation to the human immune system (van Dorp, *et al*., 2020). The G614 variant in the S protein is thought to increase infectivity and transmissibility of the virus (Plante, *et al*., 2021). Higher viral loads were reported in clinical samples with virus containing G614 compared to the previously circulating D614 (Zhang, *et al*., 2020), however, no association was made with severity of illness- measured by hospitalisation outcomes (Korber, *et al*., 2020). SARS-CoV-2 is more infectious than SARS-CoV-1, with a higher reproductive number (R_0), SARS-CoV-2 is estimated to have a reproductive number of 2·5 (range 1·8–3·6) compared with 2·0–3·0 for SARS-CoV, indicating a more efficient spread (Petersen, *et al*., 2020). This quality is attributed to structural differences in SARS-CoV-2 that enable stronger binding to ACE 2 receptors thus increasing the efficiency at which SARS-CoV-2 infects host cells compared to SARS-CoV-1 (Xia, *et al*., 2020). SARS-CoV-2 has a greater affinity for the upper respiratory tract and conjunctiva, infection of the bronchial epithelium type 1 pneumocytes is higher than SARS-CoV-

1 but similar to MERS-CoV, whereas in the conjunctiva, SARS-CoV-2 replication is greater than SARS-CoV-1 (Hui, *et al.*, 2020).

As previously described, most coronaviruses are responsible for the common cold, causing mild upper respiratory infections with occasional gastrointestinal involvement (Ye, *et al.*, 2020). In contrast, SARS-CoV-2 causes severe 'flu'-like symptoms that can subsequently progress into acute respiratory distress syndrome (ARDS), pneumonia, renal failure, and death (Polak, *et al.*, 2020). The broad nature of clinical implications can be explained by the host target cell receptor, ACE2 (Liu, *et al.*, 2021). This receptor is found in the epithelium of the lung, intestine, kidneys, and blood vessels, explaining the diverse nature of symptoms (Salamanna, *et al.*, 2020). In lung cells, replication and release of the virus can lead to non-specific symptoms such as fever, headache, myalgia, and respiratory symptoms (Cevik, *et al.*, 2020b). Animal models have shown that SARS-CoV-2 can damage the olfactory epithelium, this is a suggested cause of the temporary, but sometimes long lasting, loss of smell and taste attributed to SARS-COV-2 infections (Bryche, *et al.*, 2020). However, it is not known whether the pathological changes in these organs a direct result of viral infection, but a result of cytokine dysregulation or coagulopathy, or a multifactored response (Cevik, *et al.*, 2020). Compared to other coronaviruses, and the most closely related, SARS-CoV, the incubation period is rapid, ~5-6 days compared to 2-11 days in SARS-CoV-1 infections (Harrison, *et al.*, 2020). SARS-CoV-2 has clinical implications in not only respiratory and gastrointestinal organs (Cheung, *et al.*, 2020) but is known to have long term consequences such as myocardial inflammation (Bearse, *et al.*, 2021).

Following viral entry, the initial inflammatory response attracts virus specific T cells to the site of infection, where infected cells are eliminated before the virus spreads, leading to recovery in most people (Blanco-Melo, *et al.*, 2020). In severe disease, an aberrant host immune response is elicited, defined by low levels of type I and III interferons juxtaposed to elevated chemokines and high expression of IL-6 (Blanco-Melo, *et al.*, 2020). Post-mortem histological analysis of lung tissues of patients who have died as a result of SARS-CoV-2 infection have confirmed the inflammatory nature of the infection (Borczuk, *et al.*, 2020). Pathological features of severe infection include bilateral diffuse alveolar damage, interstitial mononuclear inflammatory infiltrates, hyaline-membrane formation, and desquamation consistent with acute respiratory distress syndrome (Schaefer, *et al.*, 2020). One of the first pathological changes to the epithelium in severe SARS-COV-2 and a defining feature of the virus is the presence of mucus plugs with fibrinous exudate in the respiratory tract which may account for the severity of SARS-COV-2 even in young adults (Wang, *et al.*, 2020). CT image studies in the pulmonary parenchymal region of SARS-COV-2 patients have shown a 64% occurrence of pathological fluid in the alveolar sacs which appears multifocal, patchy, or segmented and is distributed along broncho-vascular bundles or around sub-pleural areas (Zhao, *et al.*, 2020). Sputum volume increase and hypersecretion of mucus have been

seen in up to 40% of patients (Khan, *et al*., 2020). These plugs can lead to airway obstruction and decreased alveolar gas-exchange function, in these cases patients require mechanical ventilation to help them breathe (Chen, *et al*., 2020). The formation of mucus plugs is potentially caused by a cytokine storm, overproduction of IL-6, IL-10 and TNF-α initiates a hyperactive inflammatory response that results in mucus hypersecretion (Girija, *et al*., 2020).

The clinical outcomes of most coronaviruses are related to older age or infancy, whereas, in the case of SARS-CoV-2 there are a multitude of risk factors associated with severe disease, admission to the intensive care unit and, mortality (Lu, *et al*., 2020). These include underlying conditions such as hypertension, cardiovascular disease, chronic obstructive pulmonary disease, diabetes, obesity and malignancy (Sanyaoulu, *et al*., 2020), as well as virus related factors such as host immune response and potential cross reactive immune memory following exposure to seasonal coronaviruses (Felsenstein, *et al*., 2020). Sex of patient also plays a huge role in clinical outcomes, with men being more severely affected by SARS-CoV-2 infection than women (Takahashi, *et al*., 2020). This is thought to be attributed to the higher plasma concentration of innate cytokines and chemokines such as CXCL8 and IL-18 in men (Takahashi, *et al*., 2020). Increased levels of pro-inflammatory cytokines are related with severe pneumonia and increased ground glass opacities within the lungs (Wu, *et al*., 2020). Comparatively, women have more robust T cell activation than men (Takahashi, *et al*., 2020). In males, poor T cell response negatively correlated with age, whereas in females, this decline was not seen (Takahashi, *et al*., 2020). This is of note as both being male and older age is associated with increased risk of mortality and severe disease- over 90% of SARS-COV-2 related deaths in the UK have been in people over the age of 60, of which, 60% in men (Williamson, *et al*., 2020).

In response to the pandemic, an unprecedented response was elicited by biomedical and pharmaceutical industries with regards to finding safe and effective treatment options for SARS-CoV-2. Early efforts were focussed on the identification of pre-existing drugs that show antiviral activity against SARS-CoV-2 (Dolgin, 2021). Initially there was a plethora of known drugs with some antiviral activity against SARS-CoV-2, including chloroquine, interferons, lopinavir/ritonavir and ribavirin (Santos, *et al*., 2020). However, in the United Kingdom, only corticosteroids, low molecular weight heparin, an antiviral drug Remdesivir and monoclonal antibodies such as tocilizumab, and sarilumab have been approved for treatment (National Institute for Health and Care Excellence, 2021). Trials are ongoing with a concerted effort between research institutions and universities to generate the *in vitro* data required to provide the basis for investigation. Of these trials a number involve the use of heparin, unfractionated, low molecular weight, or heparin analogues with the hopes that its beneficial pharmacological effects as an anti-coagulator, anti-viral and anti-inflammatory can be exerted with little to no adverse effects, as seen below in Table 7 *(ClinicalTrials, 2021b)*.

Table 7: Current studies and trials underway regarding heparin as a potential treatment for SARS-CoV-2. *There are currently 59 clinical trials registered that reference heparin and SARS-CoV-2 in their registration across 22 countries (as of the 2nd of August 2021)* (ClinicalTrials, 2021b).

Title	Interventions	Phases	Status	NCT number
Coagulopathy of COVID-19: A Pragmatic Randomized Controlled Trial of Therapeutic Anticoagulation Versus Standard Care	Drug: Therapeutic Anticoagulation	Phase 3	Active, not recruiting	NCT04362085
CoV-Hep Study: Regional Anticoagulation Modalities in Continuous Venous Venous Hemodialysis in Patients With COVID-19	Drug: unfractionated Heparin	Not Applicable		NCT04487990
Intermediate-dose vs Standard Prophylactic Anticoagulation and Statin vs Placebo in ICU Patients With COVID-19	Drug: intermediate dose Enoxaparin/ unfractionated heparin|Drug: standard prophylactic dose Enoxaparin/ unfractionated heparin|Drug: Atorvastatin 20mg|Drug: Matched placebo	Phase 3		NCT04486508
A Pragmatic Randomized Controlled Trial of Therapeutic Anticoagulation Versus Standard Care as a Rapid Response to (SARS-CoV-2) COVID-19 Pandemic	Drug: Therapeutic anticoagulation	Phase 3		NCT04444700
Enriched Heparin Anti COVID-19 Trial	Drug: Heparin sodium|Drug: Placebo	Phase 1|Phase 2	Not yet recruiting	NCT04743011
Trial Evaluating Efficacy and Safety of Anticoagulation in Patients With COVID-19 Infection, Nested in the Corimmuno-19 Cohort	Drug: Tinzaparin or unfractionated heparin	Phase 2		NCT04344756
Clinical Characteristics and Outcomes of 187 Critically Ill Patients With Coronavirus Disease 2019 (COVID-19)	Other: demographic and clinical data obtained from hospital's electronic medical record.	Not Applicable		NCT04454372
A Simple Approach to Prevent Hospitalization for COVID-19 Patients	Drug: Recommended treatment schedule|Drug: Control treatment schedule	Not Applicable		NCT04854824
High Versus Low LMWH Dosages in Hospitalized Patients With Severe COVID-19 Pneumonia and Coagulopathy	Drug: Enoxaparin	Phase 3		NCT04408235
Nebulized Heparin for the Treatment of COVID-19	Drug: Heparin|Drug: 0.9%sodium chloride	Phase 4	Enrolling by invitation	NCT04723563
Nebulized Heparin for the Treatment of COVID-19 Induced Lung Injury	Drug: Heparin|Drug: 0.9% Sodium-chloride	Phase 4		NCT04397510
Nebulized Heparin in Severe Acute Respiratory Syndrome COVID-19	Drug: Heparin sodium | Drug: Enoxaparin	Phase 4	Recruiting	NCT04530578
Clinical Efficacy of Heparin and Tocilizumab in Patients With Severe COVID-19 Infection	Drug: Tocilizumab|Drug: Heparin - Therapeutic dosage|Drug: Heparin - Prophylactic dosage	Phase 3		NCT04600141
Nebulized Heparin for COVID19-associated Acute Respiratory Failure	Drug: Heparin|Drug: Placebo	Phase 2		NCT04842292
Inhaled Heparin for Hospitalised COVID-19 Patients	Drug: Unfractionated heparin	Phase 2|Phase 3		NCT04635241
Nebulised Heparin in Patients With Severe COVID-19	Drug: Nebulised unfractionated heparin (UFH)	Phase 2|Phase 3		NCT04545541
Efficacy Assessment of Methylprednisolone and Heparin in Patients With COVID-19 Pneumonia	Drug: Methylprednisolone|Drug: Heparin	Phase 3		NCT04485429
Assessing Safety, Hospitalization and Efficacy of rNAPc2 in COVID-19	Drug: rNAPc2|Drug: Heparin	Phase 2|Phase 3		NCT04655586
Steroids and Unfractionated Heparin in Critically Ill Patients With Pneumonia From COVID-19 Infection	Drug: Enoxaparin|Drug: Methylprednisolone|Drug: unfractionated heparin	Phase 3		NCT04528888
Anti-thrombotics for Adults Hospitalized With COVID-19 (ACTIV-4)	Drug: theraputic heparin|Drug: prophylactic heparin|Drug: P2Y12	Phase 4		NCT04505774
Anticoagulation in Critically Ill Patients With COVID-19 (The IMPACT Trial)	Drug: Enoxaparin sodium|Drug: Unfractionated heparin|Drug: Fondapariniux|Drug: Argatroban	Phase 4		NCT04406389
Effect of the Use of Anticoagulant Therapy During Hospitalization and Discharge in Patients With COVID-19 Infection	Drug: Enoxaparin	Not Applicable		NCT04508439
Intermediate or Prophylactic-Dose Anticoagulation for Venous or Arterial Thromboembolism in Severe COVID-19	Drug: Enoxaparin Prophylactic Dose|Drug: Heparin Infusion|Drug: Heparin SC|Drug: Enoxaparin/Lovenox Intermediate Dose	Phase 4		NCT04367831

Title	Intervention	Phase	Status	NCT Number
Early Prophylactic Low-molecular-weight Heparin (LMWH) in Symptomatic COVID-19 Positive Patients	Drug: Enoxaparin	Phase 3		NCT04492254
Hemostasis in COVID-19: an Adaptive Clinical Trial	Drug: acetylsalicylic acid | Drug: Unfractionated heparin nebulized	Phase 2		NCT04466670
Hamburg Edoxaban for Anticoagulation in COVID-19 Study	Drug: Anticoagulation Agents (Edoxaban and/or high dose LMWH) | Drug: Low dose Low molecular weight heparin or Placebo	Phase 3		NCT04542408
Nebulised Heparin to Reduce COVID-19 Induced Acute Lung Injury	Drug: Nebulised heparin	Phase 1 | Phase 2		NCT04511923
D-dimer Adjusted Versus Therapeutic Dose Low-molecular-weight Heparin in Patients With COVID-19 Pneumonia	Drug: low-molecular-weight heparin	Phase 4		NCT04584580
COVID-19 Disease and Coagulopathy: Assessment of Clotting Factor Levels in Patients With SARS-CoV-2 Infection	Drug: low molecular weight Heparin as standard of care treatment	Not Applicable		NCT04787510
Preventing COVID-19 Complications With Low- and High-dose Anticoagulation	Drug: Enoxaparin	Phase 3		NCT04345848
Tenecteplase in Patients With COVID-19	Drug: Tenecteplase | Drug: Placebo	Phase 2		NCT04505592
Prevention of Arteriovenous Thrombotic Events in Critically-Ill COVID-19 Patients Trial	Drug: Unfractionated Heparin IV | Drug: Enoxaparin 1 mg/kg | Drug: Clopidogrel | Drug: Unfractionated heparin SC | Drug: Enoxaparin 40 Mg/0.4 mL Injectable Solution	Phase 4		NCT04409834
ANTIcoagulation in Severe COVID-19 Patients	Drug: Tinzaparin, Low dose prophylactic anticoagulation | Drug: Tinzaparin, High dose prophylactic anticoagulation | Drug: Tinzaparin,Therapeutic anticoagulation	Phase 2	Recruiting	NCT04808882
Weight-Adjusted vs Fixed Low Doses of Low Molecular Weight Heparin For Venous Thromboembolism Prevention in COVID-19	Drug: Enoxaparin	Phase 4		NCT04373707
Effect of Anticoagulation Therapy on Clinical Outcomes in COVID-19	Drug: Rivaroxaban | Other: Standard Of Care (SOC)	Phase 2		NCT04416048
Anticoagulation in Patients Suffering From COVID-19 Disease The ANTI-CO Trial	Drug: Bivalirudin Injection | Drug: Standard treatment	Phase 4		NCT04445935
Australasian COVID-19 Trial (ASCOT) ADAptive Platform Trial	Drug: Nafamostat Mesilate | Biological: Convalescent plasma | Drug: Enoxaparin | Drug: Dalteparin | Drug: Tinzaparin | Drug: Aspirin	Phase 3		NCT04483960
A Simple Approach to Treat COVID-19 Patients at Home.	Drug: Recommended treatment schedule | Drug: Control treatment schedule	Not Applicable		NCT04794998
Safety and Immunogenicity Trial of Multi-peptide Vaccination to Prevent COVID-19 Infection in Adults	Biological: multipeptide cocktail	Phase 1		NCT04546841
Clinical Trial on the Efficacy and Safety of Bemiparin in Patients Hospitalized Because of COVID-19	Drug: Bemiparin	Phase 2		NCT04420299
Standard vs High Prophylactic Doses or Anticoagulation in Patients With High Risk of Thrombosis Admitted With COVID-19 Pneumonia (PROTHROMCOVID)	Drug: Tinzaparin	Phase 3		NCT04730856
Ultra Low Doses of Therapy With Radiation Applicated to COVID-19	Radiation: Ultra-Low-dose radiotherapy | Device: ventilatory support with oxygen therapy | Drug: Lopinavir/ritonavir | Drug: Hydroxychloroquine | Drug: Azithromycin | Drug: Piperacillin/tazobactam | Drug: Low molecular weight heparin | Drug: Corticosteroid injection | Drug: Tocilizumab	Not Applicable		NCT04394182
Heparins for Thromboprophylaxis in COVID-19 Patients: HETHICO Study in Veneto	Drug: Low Molecular Weight Heparin	Not Applicable		NCT04393805
Effects of Low Molecular Weight Heparin Therapy With Soft-Mist Inhaler for COVID-19 Induced Hypoxemia	Drug: Inhaled Low molecular weight heparin | Drug: Standard Treatment	Phase 2 | Phase 3		NCT04990830
Factor Xa Inhibitor Versus Standard of Care Heparin in Hospitalized Patients With COVID-19 (XACT)	Drug: Enoxaparin | Drug: Rivaroxaban	Phase 2		NCT04640181
Antithrombotic Therapy to Ameliorate Complications of COVID-19 (ATTACC)	Drug: Heparin	Phase 2 | Phase 3		NCT04372589
Intranasal Heparin Tolerability Study	Drug: Intranasal heparin sodium (porcine)	Early Phase 1		NCT04490239
Increased Risk of VTE and Higher Hypercoagulability in Patients Recovered in ICU and in Medical Ward for COVID-19	Drug: thromboprophylaxis with low-molecular-weight heparin or fondaparinux	Not Applicable		NCT04359212
Use of UC-MSCs for COVID-19 Patients	Biological: Umbilical Cord Mesenchymal Stem Cells + Heparin along with best supportive care. | Other: Vehicle + Heparin along with best supportive care	Phase 1 | Phase 2	Completed	NCT04355728
Full Dose Heparin Vs. Prophylactic Or Intermediate Dose Heparin in High Risk COVID-19 Patients	Drug: Enoxaparin | Drug: Prophylactic/Intermediate Dose Enoxaparin	Phase 3		NCT04401293
Prophylactic Versus Therapeutic Dose Anticoagulation In COVID-19 Infection at the Time of Admission To Critical Care Units	Not Applicable	Not Applicable		NCT04829552
Comparison of Two Doses of Enoxaparin for Thromboprophylaxis in Hospitalized COVID-19 Patients	Drug: Enoxaparin	Phase 3		NCT04366960
Full Anticoagulation Versus Prophylaxis in COVID-19: COALIZAO ACTION Trial	Drug: Group 1: Rivaroxaban 20mg/d followed by enoxaparin/unfractionated heparin when needed | Drug: Group 2: control group with enoxaparin 40mg/d	Phase 4		NCT04394377
Suloexide in the Treatment of Early Stages of COVID-19	Drug: Sulodexide | Drug: Placebo	Phase 2 | Phase 3		NCT04483830
Eosinophil and Anticoagulation in COVID-19 Patients	Drug: LMWH	Not Applicable		NCT04507282
Thrombosis and Covid-19	Diagnostic Test: TEM-tPA	Not Applicable		NCT04366778
Evolution of COVID-19 in Anticoagulated or Antiaggregated Patients (CORONA Study)	Other: Antithrombotic Therapy (anticoagulant and/or antiplatelet) before admission for Covid19	Not Applicable		NCT04518735
Dosing of Thromboprophylaxis and Mortality in Critically Ill COVID-19 Patients	Drug: Dose of tinzaparin or dalteparin	Not Applicable		NCT04593654
Anticoagulant Therapy and 28-days Mortality in Critically Ill COVID-19 Patients	Drug: Dose of Tinzaparin or Dalteparin	Not Applicable		NCT04412304

Chapter 2: Sulphated carbohydrates as therapeutic agents for SARS-COV-2: A Scoping Review

Background:

Many have studies investigated the role of heparin as a mediator of viral cell entry and as a potential therapeutic agent for enveloped viruses. More recently attention has been turned to the novel coronavirus, SARS-CoV-2. This research could provide a basis for further research into the commonly used and already approved anticoagulant heparin, synthetic analogues, and modified forms. Therefore, in this scoping review, an investigation was carried out into the efficacy of these compounds as a treatment for SARS-COV-2 by exploiting their pleiotropic properties for use as a multi-purpose therapy, to provide a clear basis of evidence for this direction of research.

Methods:

A systematic search of PubMed, WebofScience, Clinicaltrial.gov, medRvix and bioRvix for studies up to January 9th, 2021, on heparin as a treatment for SARS-COV-2 was carried out. Clinical trials, retrospective cohort studies, prospective cohort studies and observational studies and research papers were included investigating the therapeutic potential of heparin with regards to its anticoagulant, anti-inflammatory, and antiviral properties. Data were collected on the type of heparin used, including modified forms, dosage time and concentration and clinical and laboratory parameters involved in studies.

Findings:

45 studies were eligible for inclusion, investigating the role of the proteoglycan HSPG in the entry of SARS-CoV-2 into target cells and the use of heparin, low molecular weight heparin forms and heparin mimetics to inhibit this interaction (n=29), the anti-inflammatory action of heparins (n=7), the anticoagulant properties of heparins (n=21) and the combined effect of anti-inflammatory, anticoagulant and antiviral properties of heparins (n=3).

Interpretation:

The amount of research both investigating and evaluating heparin as a potential treatment for SARS-COV-2 is limited, with a greater focus on the anti-coagulant properties. Most importantly, the type and dosage strategy of heparin treatment, i.e., enoxaparin and prophylactic versus therapeutic, should be further researched to optimise future treatment protocols. In addition, further focus should be on the potential of the anti-inflammatory properties of heparin as the scope here is restricted by lack of research.

Research in Context:

Evidence before this study:

The impact of SARS-COV-2 has been tremendous, not only affecting the health and welfare of people, but the healthcare system, education, and the economy. With the vaccine rollout projected to be completed in late 2021, early 2022, the need for effective treatment is necessary to relieve the strain on the healthcare system. Heparin is already in use as an anticoagulant, with a wide body of research supporting its efficacy and safety, this negates the need for approval, increasing the speed at which heparin could be utilised as an antiviral. Given the impact of SARS-COV-2 and the potential use of heparin as an antiviral as revealed in the past, this potential knowledge gap was systematically reviewed by performing a scoping review on research articles investigating the use of heparins as a potential antiviral. Searching the literature in PubMed, up to the 9th of January 2021, including primary research articles, retrospective cohort studies, prospective cohort studies and observational studies in humans, which investigated the role of heparins as a mediator of viral cell entry and their antiviral, anti-coagulant, and anti-inflammatory properties.

Added value of this study:

This scoping review reports five main findings. First, that heparin can competitively inhibit HS binding of SARS-CoV2 to target host cells, independent of the angiotensin 2 receptor. Furthermore, this inhibition extends to other heparin forms such as low molecular weight heparins, modified heparins, most significantly O-sulphated heparin forms and heparin mimetics. Secondly, low molecular weight heparin or similar mimetics appear to be the most promising treatment candidates *in vivo* and *in vitro*. Thirdly, *in vivo*, treatment of SARS-COV-2 with heparin is associated with a significant reduction of mortality risk, when compared to other standard treatments such as hydroxychloroquine and remdesivir. Fourth, heparin treatment can act to modulate inflammatory responses *in vivo* with positive effects. Finally, that heparin can be used prophylactically and therapeutically with benefits in both cases.

Implications of all the available evidence to date:

This review provides evidence that heparan sulphate is implicated in the viral cell entry of SARS-CoV-2, it outlines the potential mechanisms by which this occurs and how these interactions can be inhibited by the use of a heparan sulphate proxy, heparin. Furthermore, it provides the clinical evidence to support that heparin is an effective treatment for SARS-COV-2, not only this, but the efficacy, dosage concentrations and timings of other heparin forms such as low molecular weight

heparins and mimetics have been identified. This collation of research can provide the basis for more direct research into the potential of heparin as an antiviral treatment for SARS-CoV-2.

<u>Introduction:</u>

In late 2019, a novel β-coronavirus causing severe acute respiratory syndrome (SARS-CoV-2) emerged in China and rapidly spread worldwide, causing a pandemic with global impact. On January 9th, 2021, Corona Virus Disease 2019 (SARS-COV-2), caused by SARS-CoV-2 infection, case mortality reached an all-time high of 15,603 deaths per day (World Health Organisation, 2021). Despite global efforts to identify effective interventions for the prevention and treatment of SARS-COV-2, which has resulted in 2,533 trials, completed or underway (as of the 13th of January 2021) (Cytel, 2021), evidence for effective treatment remains limited. With the emergence of new variants and a prolonged wait for protection of the whole population by vaccination and potential vaccine escape, the need for effective, accessible, and affordable treatment is paramount. Due to the urgency of the current situation, initial drug discovery should focus on repurposing licensed drugs, as the dosage information and safety information are largely to hand.

Current coronavirus treatments have focussed on repurposing pre-existing antiviral or immune modulators (Saha, *et al*., 2020), with the four most promising being remdesivir, hydroxychloroquine, lopinavir-ritonavir and Interferons. The most promising treatment was remdesivir, a nucleoside analogue prodrug, originally developed to treat hepatitis C and subsequently tested for use against Ebola (Mulangu, *et al*., 2019), albeit with disappointing results. Initial results from early in the pandemic, showed that remdesivir could reduce recovery times by up to 5 days among patients with symptom duration of 10 days (Wang, *et al*., 2020). This finding has been consolidated by other research that found remdesivir could reduce time to clinical improvement (Beigel, *et al*., 2020) and in one trial, it was found that a five-day course (not the previously tested 10-day regimen of remedesivir), showed a statistically significant reduction in clinical status compared to that of standard care (Wang, *et al*., 2020b). Despite the early hopes for this antiviral treatment, further research demonstrated that remdesivir has little to no effect on mortality or serious adverse events in patients hospitalised with SARS-COV-2 (National Institute for Healthcare and Excellence, 2020) (Goldman, *et al*., 2020).

Another candidate was chloroquine or hydroxychloroquine, both antimalarials shown to have *in vitro* activity against other viruses (Paton, *et al*., 2011) (Borges, *et al*., 2013) (Murray, *et al*., 2020). Preliminary reports of a trial investigating the efficacy of chloroquine phosphate, reported small decreases in body temperature and coughs in cases with radiological pneumonia but without severe hypoxia (Gao, *et al*., 2020). However, it was later found that the endpoints specified in the published protocol differed from those reported, the low dose group results were not included, and the trial was prematurely suspended (Singh, *et al*., 2020). Following this, the case for hydroxychloroquine

and chloroquine became anecdotal as research continued to show that it was not an effective or viable treatment for SARS-COV-2 (Self, *et al*., 2020) (The RECOVERY Collaborative Group, 2020) (Cavalcanti, *et al*., 2020). The hope for hydroxychloroquine was quashed as government agencies began to caution against its use, with the Medicines and Healthcare products regulatory Agency (MHRA) terminating all hydroxychloroquine trials in the UK (Medicines and Healthcare products Regulatory Agency, 2020).

The combination of the HIV type 1 aspartate protease inhibitor, lopinavir, with evidenced *in vitro* inhibitory activity against SARS-CoV (Chu, *et al*., 2004) and ritonavir, the cytochrome P450 inhibitor utilised to increase the plasma half-life of lopinavir (National Insititute for Health and Care Excellence, 2019), was promising. Lopinavir was identified as a potential treatment as it was found to have activity both *in vitro* (de Wilde, *et al*., 2014) and animal (Chan, *et al*., 2015) models against Middle East respiratory syndrome coronavirus (MERS-CoV). An initial study found that lopinavir and ritonavir given within 12 days after the onset of symptoms was associated with a shorter time to clinical improvement, with negligible differences in reduction of viral RNA load, duration of oxygen therapy, duration of viral RNA detectability, duration of hospitalisation and time from randomisation to death (Cao, *et al*., 2020). A second study showed that lopinavir-ritonavir is effective, but only as part of a cocktail of therapy (Hung, *et al*., 2020). Early treatment with triple antiviral therapy of interferon (IFN) beta-1b, lopinavir-ritonavir and ribavirin, alongside standard care, is not only safe, but shortens the duration of viral shedding compared with lopinavir-ritonavir alone in patients with mild to moderate SARS-COV-2 (Hung, *et al*., 2020). Further studies of lopinavir-ritonavir conclude that this treatment has no beneficial effect on 28-day mortality in hospitalised patients compared to standard care and another found there was no reduction in 28-day mortality, length of hospital stay or risk of progressing to invasive mechanical ventilation or death (RECOVERY Collaborative Group, 2020). Interferons represent the first line of defence against a wide range of viruses, inhibiting viral infection by blocking viral replication and eliminating virus-infected cells and are typically given alongside antivirals, as seen with the lopinavir-ritonavir-IFNβ1b cocktail (Baghaei *et al*., 2021).

The host innate immune response is initiated when viral products are recognised by host cell pattern receptors, including Toll-like receptors and RIG-I-like receptors (Meylan and Tschopp, 2006). This response results in the production of interferons central to combating virus infection and modulating the antiviral immune response (Lee and Ashkar, 2018). Type I IFNs consist of multiple subtypes of IFN-α and a single type of IFN-β in addition to the less well-characterised IFN-δ, -ε, -κ, -τ, -ω, and -ζ. Type I interferons are one of the first cytokines produced during a virus infection, critical for the induction of both an antiviral response within infected and target cells and activating innate immune cells that serve to control viral replication and activate the adaptive immune response (Lee and Ashkar, 2012). In contrast, Type II IFN has only one member, IFN-γ, and is predominantly

produced by natural killer cells during the antiviral innate immune response, evidence has demonstrated that type I IFN, IL-12, IL-15 and IL-18 can all stimulate type-2 IFN production (Pegram *et al*., 2010).

Following the SARS outbreak of 2002-2003, early research investigated the inhibitory effect of type I and II IFNs on SARS-CoV multiplication in cell culture, to reveal that cells infected with SARS-CoV are sensitive to IFN-α (Type 1 IFN) and IFN-γ (Type II IFN), leading to a 10-fold inhibition of virus growth (Spiegel *et al*., 2004). Interferon-β was found to be the most potent, reducing viral titres by 1000-fold (Spiegel *et al*., 2004). Interferon-β and -λ (Type III IFN) can be secreted by any type of cell upon viral infection, whereas IFN-αs are generally produced by immune cells, particularly monocytes and dendritic cells (Korthals *et al*., 2007). Clinical studies of SARS-CoV-2 found that a proportion of severe SARS-COV-2 patients had impaired type 1 interferon activity (Hadjadj, *et al*., 2020). Specifically, serum IFN-β levels were significantly lower in patients with SARS-CoV-2 than in healthy individuals (Hadjadj *et al*., 2020). Furthermore, various studies demonstrated that SARS-COV2 proteins nsp1, 6, 13, and ORF6 target INF-β to evade the host innate immune mechanisms inhibiting of IFN-β production, downstream signalling pathways and developing resistance to interferon (Lei *et al*., 2020) (Xia *et al*., 2020). Preliminary studies showed that treatment with subcutaneous IFNβ1b alone was not an effective treatment (Hung, *et al*., 2020), however, new evidence shows that patients who received nebulised IFNβ1a had significantly increased odds of clinical improvement than those who received the placebo, but no significant difference on the odds of hospital discharge (Monk, *et al*., 2020). Although a promising candidate it is clear that more research is needed before application as a standard treatment. The World Health Organisation found that these four most promising drugs had no significant impact on the reduction of inpatient mortality, the initiation of mechanical ventilation or hospitalisation duration and emphasised the necessity for identification and use of better treatments (WHO Solidarity Trial Consortium, 2020).

Heparin is a member of a family of polyanionic polysaccharides known as glycosaminoglycans. Unfractionated heparin, low molecular weight heparins and other heparin derivatives are approved for use clinically, meaning heparin is currently the second most widely used drug by weight globally (Oduah, *et al*., 2016). Heparin is important in the prevention and treatment of pulmonary embolism, unstable angina, acute peripheral arterial occlusion, thromboprophylaxis in surgical patients and during pregnancy and the prevention of thrombosis during haemodialysis (National Institute for Health and Care Excellence, 2021). Although heparin is primarily utilised for its anticoagulant properties, it is known to have pleiotropic effects, known to possess anti-angiogenesis, anti-complement, anti-inflammatory, anti-metastatic, immunomodulatory, anti-viral activity (Page, 2013). Heparin has been shown to have broad-spectrum activity against many enveloped viruses, including *coronaviridae* and SARs associated strain HSR1, in addition to alphaviruses, herpes,

HIV, flaviviruses and influenza (Conzelmann, *et al*., 2020). In addition, the closely related glycosaminoglycan, heparan sulphate, has been shown to be involved in mediating the attachment of viral surface proteins to target cells, in several viruses including coronavirus (Mycroft-West, *et al*., 2020).

In this scoping review, primary research articles, retrospective, prospective and observational cohort studies on the role heparin and like compounds as mediators of viral cell entry and as potential antiviral therapeutics were systematically reviewed. For these studies, type of heparin or sulphated compound, mechanism of action, effective dosage parameters and their efficacy *in vitro* and *in vivo* were recorded.

Methods:

The scoping review was conducted according to the Preferred reporting terms for Systematic Reviews and Meta-analyses (PRISMA) guideline (Moher, *et al*., 2009). Pubmed and WebofScience were systematically searched, the original search results were reviewed, investigating the antiviral, anticoagulant and anti-inflammatory properties of heparin and other sulphated glycosaminoglycans against SARS-CoV-2. Keywords searched were: 'Heparin, heparan sulphate AND glycosaminoglycans OR carbohydrates AND SARS-COV-2'. Randomised controlled trials (RCTs), research articles and observational and retrospective studies were eligible for inclusion. To be eligible for inclusion, RCTs had to investigate both a treatment and control group, in which the latter was allowed to vary between standard care, a specific pharmacological treatment or no pharmacological treatment. *In vitro* studies, animal studies, trials investigating different doses of the same drug, or similar drugs were included due to the urgency and novelty of the situation, meaning research was fairly limited in this area. Exclusion criteria included review articles, only primary research was accepted, one arm clinical trials and single person case studies. The search was limited to studies published from December 2019 to the 9th of January 2021.

The initial study selection was based on title and abstract, with subsequent selection based on the full text. Furthermore, BioRxiv, MedRxiv and ClinicalTrials.gov were examined for further eligible studies, following checking the preselected publications and cross-checking reference lists the relevant data were extracted from the final selection of studies, as described below.

Data Extraction:

A pre-defined data-extraction sheet, including aims and outcomes of study were used. Data were extracted on the description of glycosaminoglycan or carbohydrate used, methods and key findings. Pharmacological treatment modalities were clustered in the following groups: antiviral, anti-inflammatory, and anticoagulant mechanisms of action. Heparin forms and concentrations were grouped together to compare efficacy.

Role of the funding source:

There were no funders or sponsors that had any contribution to the study design, data collection, analysis or preparation.

Results:

The database search resulted in 861 studies and by cross checking reviews, clinicaltrial.gov (ClinicalTrials, 2021), medRxiv (medRxiv, 2021)and bioRxiv (bioRxiv, 2021), 479 studies that were in preprint stage by the time the search was performed were included. After removing duplicates, the first selection began with 1340 studies (see *Figure 10*). After screening articles based on title and abstract, 1202 studies were excluded, resulting in 138 studies eligible for the second screening based on full article assessment. Following the second screening 90 articles were excluded. As a result, 48 eligible research articles on the use of sulphated carbohydrates as a therapeutic agent in the treatment of SARS-CoV-2 were included in this scoping review.
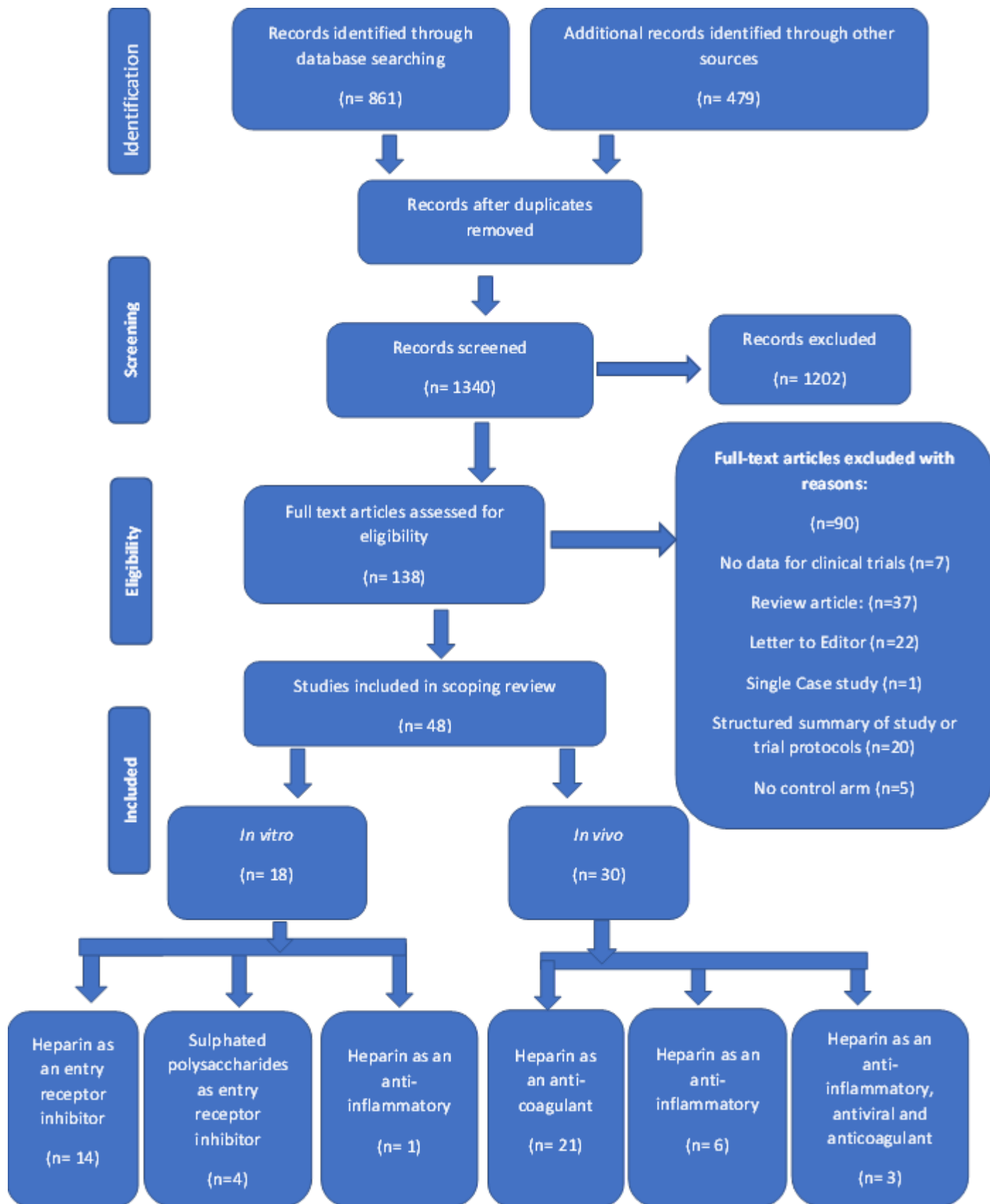
The total number of 48 included studies consisted of 18 *in vitro* studies and 30 *in vivo* studies, including 3 clinical trials, 9 observational cohort studies, 15 retrospective cohort studies and 2 prospective cohort studies. *In vitro* studies primarily focussed on the role of the proteoglycan HSPG in the entry of SARS-CoV-2 into target cells and the use of heparin, low molecular weight heparin forms and heparin mimetics to inhibit this interaction. Whereas four of these studies investigated the interaction of other sulphated polysaccharides with SARS-CoV-2 and their function-activity relationships. The final study investigates the potential role of heparin as an anti-inflammatory agent in the treatment of SARS-CoV-2. Given the pleiotropic effects of heparin, the *in vivo* studies target effects varied, with 21 investigating the use of the anticoagulant properties of heparin, 6 investigating its application as an anti-inflammatory in the treatment of SARS-CoV-2 and 3 studies utilising the anti-inflammatory, anti-coagulant and anti-viral effects of heparin.

Sulphated polysaccharides as entry receptor inhibitors:

Unfractionated heparin binds RBD:

Initial research was centred around determining the interaction of heparin, modified heparin forms and mimetics and the receptor binding domain (RBD) on the envelope protein of SARS-CoV-2. Of the research articles included in this scoping review 4 sought to elucidate this interaction.

Mycroft-West, *et al*., 2020 demonstrated that heparin binds the SARS-CoV-2 S1 protein receptor binding domain, to induce a conformational change using surface plasmon resonance and circular dichroism spectroscopy studies. This finding was confirmed and further developed with the investigation of location and affinity of heparin binding using surface plasmon resonance by, Lin Liu, 2020. The concentrations of SARS-CoV-2 proteins varied, and dilutions were two-fold, in the case of the RBD from 1100nM to 17nM and both the monomeric spike protein 446nM to 6.97nM. It was found that the RBD binds to heparin (at a concentration of 1μM) with a moderate affinity, with a K_D value of ~1μM. The full-length monomeric spike protein had a much higher affinity with a K_D value of 55nM and the trimeric spike protein showed a similar affinity with a K_D value of 64nM. This preference for the monomeric and trimeric SARS-Cov-2 spike proteins was consistent with findings by Kim, *et al*., 2020, surface plasmon resonance (SPR) direct binding assays revealed a SARS-CoV-2 spike glycoprotein (SGP) monomer and trimer (at a concentration of 50nM) had a remarkable affinity for heparin (at a concentration of 1μM), with K_D values of 40pM and 73pM, respectively. The extremely high binding affinity of SARS-CoV-2 SGP monomer to heparin could be due to the fact that the monomeric SGP spans subunit 1 which contains the proposed GAG-binding motifs 1 and 2, whilst the trimer has three of the GAG-binding sites 1 and mutated site 2 from RRAR to GSAS (Wrapp *et al*., 2020). Similarly, in the investigations carried out by Lin Liu, one of the putative heparin binding sites in the trimeric spike protein was mutated, a possible increase in strength due to multivalency may have been counteracted by a lack of a secondary binding site (Amanat *et al*., 2020). Variations in K_D values can be attributed to the different concentration of analytes used, what is of particular importance here is the overall trend of binding affinity of the different SARS-Cov-2 proteins to heparin. Unlike, Kim, *et al*., 2020 and Lin Liu, 2020, studies by Hao, *et al*., 2020 found the binding of heparin to be much weaker, like Lin Liu, 2020 it was found that RBD had the lowest affinity with a K_D value of 239.9μM, the S protein trimer and S1 subunit were weakly binding with K_D values of 16.1μM and 43.3μM respectively. The most tightly binding proteins were the full-length S protein, $K_D = 2.2$μM and S2 subunit, $K_D = 6$μM Hao, *et al*., 2020. This discrepancy could be attributed to differences in materials, investigations by both Kim *et al.* and Lin Lui, biotinylated heparin molecules were immobilised on the surface of CM5

sensor chips, whereas Hao *e al.* conversely immobilised the protein on chips. Furthermore, structural variability between both the S proteins and heparin molecules, especially those from different sources as in this case may lead to differences. Further exploration of the specificity of binding by Partridge, *et al.*, 2020, revealed the polybasic furin cleavage site in S1S2 as a potential binding site. Investigation using a mutated S1S2 protein lacking this site showed that mutated S1S2 and RBD had a significantly lower affinity for both UFH (SARS-CoV-2 spike S1S2 EC_{50}= 217.8nM, RBD EC_{50}=818.4nM) and dalteparin (EC_{50} SARS-CoV-2 spike S1S2= 162.2nM and RBD EC_{50} = 288.3nM) compared to wild type (SARS-CoV-2 spike S1S2 EC_{50} =6.8nM and RBD= 9.3nM).

Mycroft-West, *et al.*, 2020b observed that significant decreases were seen in the number of plaque forming units upon heparin treatment for both SARS-CoV and SARS-CoV-2 isolates, the latter was more susceptible to inhibition, 80%, than the former ~60%. Cells treated with heparin also inhibited SARS-CoV-2 RBD binding to the cell surface of Vero cells. In both cases, the concentrations used in these assays cover the prophylactic and therapeutic concentrations used in nebulised heparin protocols (two-fold dilutions from 200µg/mL to 6.25µg/mL). The inhibitory activity of prophylactic and therapeutic doses was confirmed by, Partridge, *et al.*, 2020, finding that using 100nM of SARS-CoV-2 S1S2 inhibition of RT4 cell binding by UFH was inhibited at a concentration of 0.033U.ml^{-1}. This is significantly lower than the target prophylactic and therapeutic concentrations of UFH in serum of 0.1-0.4U.ml^{-1} and 0.3-0.7U.ml^{-1} respectively.

Similarly, Clausen, *et al.*, 2020, explored the role of heparin in inhibition of GFP-expressing VSV S protein pseudotyped virus binding studies, assessed qualitatively by fluorescence microscopy and quantitively by flow cytometry and found that UFH reduced infection in Vero cells potently, by more than ~4 fold at 0.5µg.ml-1 and at higher concentrations. Studies were extended to a clinically relevant strain of SARS-CoV-2, USA-WA1/2020, with heparin shown to be a potent inhibitor of binding and infection. In addition, studies revealed that unfractionated heparin reduced infection in primary human bronchial epithelial cells by more than 5-fold, with little effect on cell viability (Clausen, *et al.*, 2020). In contrast to these studies, Gasbarri, *et al.*, 2020 found that unfractionated heparin had no inhibitory activity up to 1000µg.ml^{-1}. The authors do suggest that the discrepancy in their data with existing literature could be due to differences in the clinical isolates used and that in Vero-E6 cells, as used in this study, are abundant in ACE2 decreasing the dependency of the virus on HS (Gasbarri, *et al.*, 2020).

The interaction between RBD and heparin was confirmed by Qi Zhang, 2020, in ACE2-GFP HEK293T cells. A luciferase assay showed that heparin was found to dose-dependently mitigate luciferase expression from both SARS-CoV and CoV-2 PP with little impact on cell viability. This inhibition was more significantly seen in SARS-CoV-2 transduced human lung epithelial Calu-3

cells, which is of value given SARS-CoV-2 affects the respiratory system. Similarly, a heparin pull-down assay showed that the purified spike ectodomain readily bound to heparin conjugated beads, furthermore, this binding was sustained when exposed to salt concentrations found on the airway surface.

These studies show that heparin can act as an inhibitor prophylactically- as shown by plaque forming assays Mycroft-West, *et al*., 2020 and therapeutically, cell binding assays Mycroft-West, *et al*., 2020. Not only that but this interaction is more potent in human lung epithelial Calu-3 cells, when compared to ACE2-GFP HEK293T overexpressing cells and is sustained under physiological conditions similar to that found in the airway.

Structural studies of RBD-heparin binding:

To elucidate whether the binding of heparin and SARS-CoV-2 produces conformational changes in the SARS-CoV-2 RBD a structural study was carried out Mycroft-West, *et al*., 2020. Using circular dichroism (CD) spectroscopy, it was found that heparin binds and induces conformational change in the way of a 1.5% increase in α-helix content and a 2.1% decrease in global β-sheet. Demonstrating that under aqueous conditions of physiological relevance a conformational change occurs upon heparin and SARS-CoV-2 RBD binding. To ensure these changes were not seen as a result of the addition of heparin alone, as in high concentrations heparin can possess a CD spectrum, a theoretical spectrum was produced by the addition of spectra of the SARS-CoV-2 S1 RBD and heparin. This theoretical spectrum differed from the experimental spectra, serving to demonstrate that the change in the CD spectra, thus the conformational change, is a result of binding to heparin.

Other heparin forms binding to heparin:

The structural requirements of the interaction between SARS-CoV-2 S1 RBD and heparin have been examined to determine the optimal and minimal sulphation level and size required for binding to occur.

Size and sulphation level of heparins:

Length of heparin chain
A series of surface plasmon binding assays were carried out using a library of heparin derivatives and size defined fragments to investigate the relationship between size and sulphation level by, Mycroft-West, *et al*., 2020. Chain length analysis showed that showed that shorter chain oligosaccharides were not effective binding inhibitors, with the longer chains also showing little activity, a heparin-derived octasaccharide had no binding activity, whereas a decasaccharide showed modest inhibition of SARS-CoV-2 S1 binding to immobilised heparin at 0. 17μg.ml^{-1}.

However, it is unclear if this lack of activity is an artefact of the manufacturing processes used. The antiviral activity of a number of UFHs revealed that activity appeared to correlate broadly with their molecular weight, with the highest molecular weight heparins 15,650-19,100Da being more potent inhibitors than LMWHs, 4200-6650Da Tree, *et al*., 2020.

However, analysis using CD spectroscopy showed that a hexasaccharide fraction could induce similar conformational changes to heparin, despite the lack of inhibitory activity shown in SPR binding studies Mycroft-West, *et al*., 2020. The effectiveness of a hexasaccharide was further consolidated by the identification of a synthetic, small, non-sugar highly sulphated compound known as, SPGG by screening a library of natural di-, tetra-, and hexasaccharides, Tiwari, *et al*., 2020, that was shown to be an effective inhibitor of viral entry. Lin Liu, 2020 further investigated the role of chain length and heparin binding using protein microarrays with similar results. It was found that an octasaccharide and hexasaccharide of the same tri-sulphated repeating units, (IdoA2S-GlcNS6S), exhibiting the strongest binding to the RBD, the monomeric spike protein and the trimeric protein. Length is an important factor here as a tetrasaccharide with the same repeating units had very little binding, this lack of activity was similarly shown in a tetrasaccharide of the same units. Lin Liu, 2020 further investigated the interaction of the octasaccharide with the highest binding activity using SPR to find that the IC_{50} values for the spike protein and RBD were 38nM and 264nM respectively.

Another study, Hao, *et al*., 2020, showed that optimal binding occurred with an octasaccharide in the case of the full-length S protein and its trimer. However, increased inhibitory activity was seen in less sulphated heparin fractions, tetrasaccharides to octasaccharides with regards to the full-length S protein. The inhibitory activity of smaller heparin derivatives is seen again in, Hao, *et al*., 2020, with shorter chain heparin oligosaccharides having comparable or better binding properties, with one tetrasaccharide having significantly better binding properties than its longer- up to octasaccharide, counterparts. It is evident that there is no clear dependence on size for SARS-CoV-2 S1 RBD binding, the interaction is more complex.

Level of sulphation and sulphation pattern:
Competition binding studies using SPR for chemically modified derivatives by Kim, *et al*., 2020, demonstrated that N-sulphation, 2-O-sulphation and 6-O-sulphation of heparin are all independently necessary for binding to SARS-CoV-2 SGP. SPR solution competition studies revealed that none of these modified compounds were able to compete with immobilised heparin for binding of SARS-CoV-2 SGP. However, further research has not confirmed this lack of activity upon N-de-sulphation, with studies finding that N-de-sulphation decreases inhibitory activity but does not abrogate it. The role of N sulphation in the binding of SARS-CoV-2 to heparin was further investigated by, Tandon, *et al*., 2021, it was found that selective N-de-sulphation decreased

inhibitory activity of both unfractionated heparin and enoxaparin, similar to previous SPR results Kim, *et al*., 2020. This finding is consolidated by, Mycroft-West, *et al*., 2020, showing that de-N-sulphated/ N- re-acetylated heparin maintains some inhibitory activity, however much less than unmodified heparin, with inhibitory activity at 0.05mg.ml^{-1} and 0. 17mg.ml^{-1}.

The importance of O-sulphation has been demonstrated by a number of studies, but the role of 3-O-sulphation has only been identified in one, Tiwari, *et al*., 2020, using 3-O-sulphated HSPGs and competition binding studies to reveal an integral role of 3-O-sulphation in SARS-CoV-2-SGP cell to cell fusion. Interestingly, Clausen, *et al*., 2020, explored MST-heparin, derived from a murine mastoctyoma that lacks the 3-O-sulphate group. The lack of 3-O-sulphation was found to have little effect on its inhibition of spike protein binding, with an IC$_{50}$ of 0.12 μg.ml^{-1} and 0.03μg.ml^{-1} in H1299 and A549 cells respectively.

Analysis by Mycroft-West, *et al*., 2020 into the pattern of sulphation and inhibitory activity showed that SARS-CoV-2 S1 may favour regions that are 2-O and 6-O sulphated, singly de-O-sulphated heparins at C-2 and C-6 had no detectable inhibitory activity. An analysis of the effect of heparin sulphation patterns carried out by Hao, *et al*., 2020, demonstrated that 6-O sulphation plays a crucial role in in binding, with the addition of sulphate to the 6-O position of the glucosamine residues gradually increased the binding of heparin with SARS-CoV-2-RBD. In contrast to this, Tandon, *et al*., 2021, using SPR found that selective de-sulphation at the 6-O position of GlcN in both heparin and enoxaparin did not significantly reduce the inhibitory activity. Proton nuclear magnetic resonance analysis was used to confirm the successful de-sulphation of these samples indicating that 6-O-sulphation is not required for anti-SARS-CoV-2 activity in a pseudotyped transduction model Tandon, *et al*., 2021. Further sulphation pattern analysis by, Lin Liu, 2020 confirmed the importance of 2-O sulphation, showing that when hexasaccarides were 2-O-desulphated their ability to bind both the RBD and the SARS-CoV-2 S1 protein was lost. In the case of 6-O desulphation, Lin Liu, 2020, found that that there was still binding activity, unlike Mycroft-West, *et al*., 2020, although substantially reduced.

Despite this, Mycroft-West, *et al*., 2020, found that doubly de-sulphated heparins showed modest inhibitory activity at 0. 17μg.ml^{-1}, one of which was also shown to induce very similar conformational changes to the secondary structure of SARS-CoV-2 S1 as heparin. Completely desulphated heparin showed no inhibitory activity consistent with other SPR results, Tandon, *et al*., 2021, whereas over-sulphated heparin was the most inhibitory heparin derivative but was not as effective as native heparin Mycroft-West, *et al*., 2020.

The role of N sulphation in the binding of SARS-CoV-2 to heparin was investigated by, Tandon, *et al*., 2021, selective N-de-sulphation decreased inhibitory activity of both unfractionated heparin and enoxaparin, consistent with previous SPR results Kim, *et al*., 2020. This finding is consolidated by,

Mycroft-West, *et al.*, 2020, showing that de-N-sulphated/ N- re-acetylated heparin maintains some inhibitory activity, however much less than unmodified heparin, with inhibitory activity at 0.05mg.ml^{-1} and 0. 17mg.ml^{-1}.

Monosaccharide composition:

The importance of monosaccharide composition was investigated by Lin Liu, 2020, structure-binding data that shows the replacement of a single IdoA2S unit by a GlcA unit substantially decreases binding activity, further replacement of IdoA2S abolishes any binding activity. Lin Liu, 2020, conclude the monosaccharide IdoA2S is crucial for heparin binding, however, Hao, *et al.*, 2020 found that the binding for SARS-CoV-2 RBD appears to be unaffected by monosaccharide composition, but more on the level of sulphation. The most sulphated heparin derivatives (>1 sulphate group per monosaccharide) exhibited the most binding activity and decreases proportionally with the reduction in sulphate groups. Oligosaccharides with lower sulphation levels, <1 sulphate group per monosaccharide unit, have lower or almost no inhibitory activity, consistent with Mycroft-West, *et al.*, 2020 and Kim, *et al.*, 2020.

The anticoagulant properties of heparin can be removed by periodate oxidation, which oxidises the vicinal hydroxyl groups in the glucuronic acid units of the chain, resulting in split-glycol heparin. Clausen, *et al.*, 2020, explored the effects of split-glycol heparin on the binding activity of S protein to H1299 (an adenocarcinoma cell line derived from type 2 alveolar cells) and A549 (another type 2 alveolar adenocarcinoma cell line) cells, yielding IC_{50}s of $0.04\mu\text{g.ml}^{-1}$ and $0.01\mu\text{g.ml}^{-1}$ respectively. Comparatively in the same cell lines, unfractionated heparin had IC_{50} values of $0.03\mu\text{g.ml}^{-1}$ and $0.01\mu\text{g.ml}^{-1}$ respectively.

Heparin sources:

The antiviral activity of different unfractionated heparins was investigated by (Tree, *et al.*, 2020), with preparations from Wockhardt- porcine mucosa, Celsus-porcine mucosa, Calbiochem- bovine lung and NIBSC- bovine mucosa. The antiviral activity of these preparations fell within the range of $25\text{-}41\mu\text{g}\cdot\text{ml}^{-1}$. There was no significant difference between the IC_{50} values of preparations from porcine mucosa: Celsus and Wockhardt, bovine sources: Calbiochem and NIBSC. The effect of species on the antiviral activity of UFHs determined there was no significant difference between the species. The activity of unfractionated heparin and low molecular weight heparins from the two mammalian sources was compared finding that the antiviral activity of porcine mucosa is significantly greater than its LMWH counterpart.

Low Molecular Weight Heparins:

Mycroft-West, *et al.*, 2020 investigated the binding of 800nM SARS-CoV-2 S1 RBD to enoxaparin and found that a small inhibition of binding was observed at $17\mu\text{g.ml}^{-1}$ with maximal inhibition, 70%, shown at $1.7\mu\text{g.ml}^{-1}$. Despite showing inhibitory activity, enoxaparin was found to be much

less effective of an inhibitor of SARS-CoV-2 S1 to immobilised heparin, with UFH being 30-fold more effective than enoxaparin. Contrastingly, Gasbarri, *et al*., 2020 found that enoxaparin had no inhibitory activity up to $1000\mu g.ml^{-1}$, however, this is inconsistent with other studies, showing that although enoxaparin shows inhibitory activity, it is much less than that of unfractionated heparin (Tandon, *et al*., 2021) and (Kim, *et al*., 2020).

Bermejo-Jambrina, *et al*., 2020 screened a number of low molecular weight heparins, Tinzaparin, dalteparin, enoxaparin and nadroparin. It was found that all LMWHs blocked SARS-CoV-2 binding to Huh 7.5 cells, with a level of inhibition similar to that of unfractionated heparin. Additionally, the low molecular weight heparins, inhibited infection of Huh 7.5 cells with SARS-CoV-2 pseudovirus in a dose dependent manner. Tinzaparin was the most effective at inhibiting binding at a concentration of 100IU, whereas dalteparin was found to be the most effective at inhibiting infection transmission at 50IU. Enoxaparin showed a consistently high level of inhibition for binding and inhibition, whereas Nadroparin showed the least inhibitory activity. Enoxaparin was further studied due to its high level of activity. It has been found that epithelial cells expressing ACE2 are primary target cells for SARS-CoV-2 infection in the lung and intestinal tract, to investigate the role of this in comparison to HS proteoglycans studies were carried out using antibodies against ACE2 and enoxaparin and unfractionated heparin. It was demonstrated that enoxaparin blocked infection of Caco2 cells to a similar level as antibodies against ACE2 and combining ACE2 with heparin or exoxaparin did not increase the inhibition seen. Further studies into different cell lines showed that enoxaparin inhibited infection of primary alveolar macrophages with SARS-CoV-2 using Langerhans cells and dendritic cells (Bermejo-Jambrina, *et al*., 2020).

Mimetics:

Following identification of 3-O-sulphation as a key component for cell binding, the activity of the heparin sulphated pentagalloylglucopyranoside (SPGG) was investigated by Tiwari, *et al*., 2020. The effect of SPGG on cell-to-cell fusion was studied in both CHO-K1 cells and human HEK293T, with SPGG effectively inhibiting cell to cell fusion in both cell lines effectively with an IC_{50} in both cell lines of approximately $1\mu M$. Their studies also identified that overexpression of 3-O-sulphated heparan sulphates contribute to fusion of SARS-CoV-2. To test this selectivity, fondaparinux, a synthetic pentasaccharide with a central 3-O-sulphated glucosamine was used. It was shown that fondaparinux could inhibit SARS-CoV-2-SGP binding with target cells expressing 3-O-sulphated heparan sulphate in a dose dependent manner. At a concentration of $100\mu M$, fondaparinux reduced cell to cell fusion to the basal levels observed for unmodified HSPGs. Yang Yang, 2020 investigated the binding activity of a heparin oligomer (eicosasaccharide) and fondaparinux using native mass spectrometry. It was found that addition of fondaparinux results in a noticeable shift of the ionic signal, that correlates with the mass of fondaparinux. Importantly only 1:1 fondaparinux-RBD complexes were observed, similarly the heparin eicosasaccharide only bound to RBD in a 1:1 ratio.

Molecular dynamic simulations of the fondaparinux-RBD complex indicates that the binding results in significant conformational changes on the surface of the RBD. The binding interaction of fondaparinux was investigated by, Hao, *et al*., 2020 using SARS-CoV-2 RBD, S protein trimer, S1 subunit, S2 subunit and full-length S protein. The results showed K_D values similar to long chain heparin, RBD= 10.3μM, S protein trimer= 28.3μM, S1 subunit= 25.1 μM, S2 subunit= 3.5μM and full-length S protein= 13.9μM. Despite this similarity, there is a subtle difference, the affinity for the S1 subunit and RBD to fondaparinux is much more similar. In contrast, Partridge, *et al*., 2020, found that fondaparinux had no effect on SARS-CoV-2 S1S2 binding activity to RT4 cells at concentrations up to 0.1mg.ml^{-1}, despite having a therapeutic concentration of <2μg.ml^{-1}.

A HS mimetic, pixatimod, has been identified as an attractive candidate for SARS-CoV-2, with immunomodulatory and heparanase inhibiting properties, extensive study by Guimond, *et al*., 2020 found pixatimod to be a potent inhibitor of SARS-CoV-2. Circular dichroism studies revealed that not only did pixatimod bind to SARS-CoV-2 EcS1-RBD (expressed by *Escherichia coli* but induced conformational changes, with decreased α-helical content upon pixatimod binding, compared to increased α-helical upon heparin binding, consistent with Mycroft-West, *et al*., 2020. In both cases, heparin and pixatimod decreased global β-sheet content and caused increases in turn structure. The effects of pixatimod on protein stability were investigated using differential scanning fluorimetry (DSF), revealing that binding of pixatimod induced a significant decrease in melting temperature, 9.1°C, suggesting a notable destabilisation of mammalian expressed SARS-CoV-2-RBD. Strikingly, an equivalent dose of heparin resulted in a small shift of a side peak by 5.6°C, indicating partial destabilisation. An inhibition assay was carried out utilising His-tagged EcS1-RBD to monkey Vero cells- which express HSPGs and the ACE2 protein receptor involved in protein attachment, at a concentration of 100μg/mL pixatimod inhibited binding by 32%, comparatively heparin inhibited binding by 51%. The direct effects of pixatimod on the interaction of SARS-CoV-2-S1-RBD with the ACE2 protein receptor was measured using a competitive ELISA assay. It was found that pixatimod was a potent inhibitor of binding with an IC_{50} of 10.1μg.ml^{-1}, comparatively, heparin had an IC_{50} of 24.6μg.ml^{-1}. Plaque reduction neutralisation assays were carried out in Vero cells to explore the effect of pixatimod on a clinical isolate of SARS-CoV-2 (VIC01). Following pixatimod treatment significant decreases were seen in the number of plaque forming units (PFU), the analysis of multiple dose response curves gave an EC_{50} value in the range of 2.4-13.8μg.ml^{-1}. To ensure that these antiviral effects were relevant for other clinical strains assays were conducted using the isolate DE-Gbg20, with an EC_{50} value of 2.7μg.ml^{-1}. Cytotoxicity assays revealed that for Vero cells pixatimod reduced the viability of cells by 50% (CC_{50}) at a concentration of >236μg.ml^{-1}, analysis for three other isolates, DE-Gbg20, QLD02 and QLD935 yielded EC_{50} values of 0.8-11.6, 10.6 and 0.9μg.ml^{-1} respectively.

Inhibitory concentrations of heparin and heparin forms:

The potency of the inhibitory activity of heparin and other heparin forms has been quantified in number of studies by calculation of IC50 values. Clausen, *et al*., 2020, explored the inhibitory activity of unfractionated heparin, split-glycol heparin, and MST heparin against SARS-CoV-S to H1299 and A549 cells. This revealed heparin to be the most potent inhibitor of cell binding, with IC50s of: 0.03µg.ml-1 and 0.01µg.ml-1 in H1299 and A549 cells respectively. Interestingly all heparin forms demonstrated more inhibitory activity in A549 cells, with MST heparin indicating higher sensitivity to different cell lines. Split glycol heparin and MST heparin yielding IC_{50} values of 0.01µg.ml-1 and 0.03µg.ml-1 respectively, compared to 0.04µg.ml-1 and 0.12µg.ml-1 in H1299 cells.

***Table 8: Heparin and other heparin forms tested in vitro and the most inhibitory form of heparin**, adapted from (Clausen, et al., 2020), (Gasbarri, et al., 2020), (Guimond, et al., 2020), (Kim, et al., 2020), (Mycroft-West, et al., 2020), (Partridge, et al., 2020), (Tandon, et al., 2021), (Tiwari, et al., 2020), (Tree, et al., 2020) (Yang Yang, 2020) T: tinzaparin, D: dalteparin, E: enoxaparin, UFH: unfractionated heparin, MH: modified heparin forms, MSTH: MST heparin (lacks 3-O sulphate group and anti-coagulant activity), SGH: split glycol heparin (renders heparin non-anticoagulant by disrupting antithrombin binding region), NACH: non anticoagulant low molecular weight hp, Tris HS: Tri sulphate heparan sulphate*

Author (reference)	Most Inhibitory form of Heparin (including mimetics)	IC50 value most inhibitory form	Other heparin forms with inhibitory activity	Other heparin forms without inhibitory activity
(Yang Yang, 2020)	UFH	N/A	F	N/A
(Tandon, et al., 2021)	UFH	5.9µg.l^{-1}	E, MH	N/A
(Tree, et al., 2020)	UFHs	25 and 41 µg·ml^{-1}	T, D, F	N/A
(Tiwari, et al., 2020)	SPGG	N/A	F, MH	N/A
(Mycroft-West, et al., 2020)	UFH	N/A	E, MH	MH
(Gasbarri, et al., 2020)	N/A	N/A	N/A	UFH, E
(Guimond, et al., 2020)	Pixatimod	10.1 µg·ml^{-1}	UFH	N/A
(Clausen, et al.,	UFHs	0.01-0.03	MSTH, SPH	N/A

Tandon, *et al*., 2021 tested pseudotyped SARS-CoV-2 spike glycoprotein (pLV-SGP) transduction rates with inhibitor concentrations ranging from 500mg/l to 5µg/l. Both UFH and de-6-sulphated UFH gave very low IC_{50} values of 5.99µg/l (*Table 1*) and 1.77µg/l respectively. With the IC_{50} of UFH being equivalent to a concentration of ~400pM, which is 10-fold more than K_D measurements of UFH to SARS-CoV-2 SGP by SPR by Kim, *et al*., 2020. Further studies by Partridge, *et al*., 2020, showed that 10U/ml inhibited 80% of 330nM SARS-CoV-2 S1S2 interaction with cells and was significantly reduced compared to untreated controls. Competition binding studies by Kim, *et al*., 2020, using heparin, tri-sulphated non-anticoagulant heparan sulphate (TriS HS) and low molecular weight heparin synthesised from dalteparin (NACH) against SARS-CoV-2 SGP. It was revealed that, heparin had the most affinity for SARS-CoV-2-SGP with an IC_{50} of 0.056µM (*Table 1*) and TriS HP and NACH had lower affinities with IC_{50} values of 0.12µM and 26.4µM respectively.

Furthermore, it was found that enoxaparin and de-6-sulphated enoxaparin have significantly less inhibitory activity, with IC_{50} values of 1.08mg/l and 5.86mg/l respectively (Tandon, *et al*., 2021). This contradicts their previous SPR data, as 6-O-desulphation resulted in a marked decrease in inhibitory activity but is consistent with data as seen in Mycroft-West, *et al*., 2020 and Hao, *et al*., 2020.

A series of plaque formation assays were carried out by Tree, *et al*., 2020 using the LMWH Innohep (tinzaparin sodium), Clexane (enoxaparin) and preliminary exploratory experiments with Fragmin (dalteparin sodium). Innohep and Clexane had an inhibitory effect on the growth of SARS-CoV-2, with geometric mean IC_{50} values of 3.7 and 7.8mg·ml^{-1}, respectively. Exploratory experiments showed Fragmin had antiviral activity with an IC_{50} value of 3.4 mg·ml^{-1}. The inhibitory effect of

enoxaparin and dalteparin on SARS-CoV-2 S1S2 binding in RT4 cells was investigated. This revealed as above (Tree, *et al*., 2020), that these LMWHs are less potent than UFH and act only as partial inhibitors, with IC$_{50}$ values of 0.072U.ml^{-1}and 0.558U.ml^{-1} respectively. This data revealed that dalteparin could be used prophylactically for inhibition of viral infection as typical prophylactic and therapeutic serum concentrations of LMWH are 0.2-0.5IU ml^{-1} and 0.5-1.2IUml^{-1}respectively.

Heparin compared to other treatment options:

The impact of low molecular weight heparin on the outcome of patients with severe acute respiratory syndrome coronavirus 2 pneumonia, was investigated by, Falcone, *et al*., 2020, in a prospective observational study. The study sought to elucidate, the efficacy of LMWH compared to other standard treatments such as hydroxychloroquine and remdesivir. The study consisted of 315 patients, of which, 77.5% received LMWH treatment. The LMWH dosage was defined as prophylactic if subcutaneous enoxaparin 40-60mg daily was administered and therapeutic if the dosage was repeated twice daily. Multivariate analysis revealed that LMWH was associated with reduced risk of death, with a hazard ratio of 0.36. With regards to composite end point (death or severe acute respiratory distress syndrome (ARDS)), LMWH treatment remained a protective factor, with a hazard ratio of 0.61, significantly LMWH had the lowest hazard ratio of all assessed treatments.

Other sulphated polysaccharides:

Despite the fact that they are not licensed for clinical use, the potential for other sulphated polysaccharides to inhibit the binding of SARS-CoV-2 RBD has been under preliminary investigation. The scope of this research is extremely limited, with only one paper investigating the conformational changes of SARS-Cov-2 S1 RBD when bound to sulphated GAGs such as chondroitin sulphate A and C and the non-sulphated hyaluronic acid, (Mycroft-West, *et al*., 2020b). Three studies, Song, *et al*., 2020, Jang, *et al*., 2021 and Jin, *et al*., 2020 investigate the potential of marine plant derived sulphated polysaccharides to inhibit SARS-CoV-2 RBD binding to heparin *in vitro*. All studies show a potential role for these sulphated polysaccharides as an antiviral treatment in the case of SARS-CoV-2. These preliminary studies provide a basis of evidence, albeit limited, for the further research targeted towards non-traditional polysaccharide sources as potential therapeutic agents, particularly for infectious disease.

Preliminary research utilising a broad range of GAGs has revealed conformational changes in the SARS-CoV-2 S1 RBD upon binding. Studies showed that heparin decreased SARS-CoV-2 S1 RBD antiparallel content but increased helix and turn content.

Table 9: The effect of various sulphated and non-sulphated glycosaminoglycans on the secondary structure of SARS-CoV-2 S1 RBD, adapted from (Mycroft-West, et al., 2020b).

Compared to heparin, dermatan sulphate increased the helix and turn content and decreased the antiparallel secondary structure content. In contrast, chondroitin sulphate A had very similar effects on the secondary structure to heparin, with only a marginal increase in antiparallel structure and similarly marginal decreases in helix content compared to heparin. Chondroitin sulphate C increases α-helix content decreases β-antiparallel content and marginally increases β-turn content of SARS-CoV-2 S1 RBD. Chondroitin sulphate D increases helix content decreases antiparallel and marginally increases turn percentage. Hyaluronic acid slightly increases helix content, antiparallel content and turn content. In other studies, Guimond, *et al*., 2020 and Mycroft-West, *et al*., 2020,

Glycosaminoglycan	Effect on % secondary structure content				
	α-Helix	β-antiparallel	β -parallel	β-turn	Others
Heparin	↑	↓	-	↑	↓
Heparan sulphate	-	↓	-	↓	↑
Dermatan sulphate	↑	↓	-	↑	↑
Chondroitin sulphate A	↑	↓	-	↑	↓
Chondroitin sulphate C	↑	↓	-	↑	↑
Chondroitin sulphate D	↑	↓	-	↑	↑
Hyaluronic acid	↑	↓	-	↑	↓

whereby structure-activity relationships were determined, it was found that decreased global β-sheet and increases in turn structure were common between both pixatimod and heparin- both of which inhibited SARS-CoV-2 RBD binding. Extrapolating from this data, it could be concluded

that all the GAGs with the exception of heparan sulphate have the potential to inhibit binding. These results, although promising, only show structural changes and further research is required to determine whether these structural changes have any effect on the function and activity of the SARS-CoV-2 S1 RBD.

The inhibitory effects of 3 marine plant derived polysaccharides, sea cucumber sulphated polysaccharide (SCSP), fucoidan and iota-carrageenan (ι-carrageenan) and chondroitin sulphate C on SARS-CoV-2 in Vero cells was investigated by, Song, *et al*., 2020. Of the four polysaccharides screened, SCSP, showed the highest antiviral activity with IC_{50} of 9.10 µg.ml^{-1}. The other plant derived polysaccharides, fucoidan and ι-carrageenan also showed inhibitory activity at concentrations of 15.6µg.ml^{-1} and ≥125 µg mL^{-1}, respectively. It was found that CSC showed no competitive binding to the spike protein, despite inducing conformational change, as found by Mycroft-West, *et al*., 2020b.

Table 10: Marine plant derived polysaccharides with corresponding sulphate content and inhibitory concentrations, adapted from (Song, *et al*., 2020).

When considering the marine plant derived polysaccharides, an association is seen between a higher sulphation content and increased inhibitory activity, as seen in *Table 10*. The antiviral activity of

Polysaccharide	Sulphate content (%)	Inhibitory concentration (µg.ml^{-1})
SCSP	25.8 ± 2.4	9.1
Fucoidan	22.8 ± 0.7	15.6
Carrageenan	10.4 ± 0.5	≥125
CS	20.4 ± 0.3	-

SCSP was further evaluated using pseudotype virus with S glycoprotein, it was found that SCSP demonstrated significant inhibitory activity against pseudotyped virus at both 100 and 1000µg.ml^{-1}.

Further study by Jang, *et al*., 2021, evaluated the potential of lambda-carrageenan (λ-carrageenan) as an antiviral for SARS-CoV-2. Cell entry assays using pseudotyped SARS-CoV-2 in 293T cells demonstrated the ability of λ-carrageenan to suppress entry of SARS-CoV-2 in a dose dependent

manner. Following this, Vero cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.02 and treated with increasing concentrations of λ-carrageenan using remdesivir as a control. was found that λ-carrageenan not only inhibited viral infection effectively without effecting cell viability, but did so at a lower concentration than remdesivir, with an EC_{50} of $0.9 \pm 1.1 \mu g.ml^{-1}$ compared to EC_{50} of $23.5 \pm 1.2 \mu M$. λ-carrageenan was found to have a much higher selectivity index, > 333.3 compared to >12.8 in the case of remdesivir. Analyses of western blot and quantitative RT-PCR showed reduction in both viral protein in cell lysate and viral RNA level in culture supernatants by λ-carrageenan, furthermore reinfection of culture supernatant into fresh Vero cells, confirmed decreases in infectious viral titres in the presence of λ-carrageenan. These findings are consistent with, Song, *et al*., 2020, suggesting the potential for carrageenan's as antiviral treatments.

The interactions between pseudotyped particles and polysaccharides from *Saccharina japonica* were investigated by Jin, *et al*., 2020 to determine their possible inhibitory activities on the interaction between SARS-CoV-2 SGPs and ACE2. A number of differently charged and sized polysaccharide fractions were produced and screened for inhibitory activity. It was found that sulphated galactofucan (SJ-D-S-H) and glucuronomannan (Gn) strongly inhibited interactions between SARS-CoV-2 SGPs and heparin, with IC_{50}s of 27nm and 231nm respectively. Whilst displaying no obvious inhibitory activity towards the interaction between SARS-CoV-2 SGPs and ACE2. Furthermore, other high molecular weight fractions of *Saccharina japonica* had very strong inhibitory activities, decreasing proportionally with the level of charge, when compared to corresponding low molecular weight components, the same pattern in activity was still observed. The importance of sulphation content was also examined, consistent with Song, *et al*., 2020, overly sulphated polysaccharides displayed more inhibitory activity than their under-sulphated counterparts. SPR results revealed that the largest molecular weight fraction, ST-100K, an acid stable fraction, had the strongest inhibitory activity, further analysis was undertaken to determine the effect of acid sensitivity on inhibitory activity, results showed that acid-stable fractions were more inhibitory than the less stable oligosaccharides. This data shows a correlation between higher molecular weight and sulphation level and proportionally increased inhibitory activity towards SARS-CoV-2, which does not agree with data with heparin or heparin oligosaccharides.

Heparin as an anti-inflammatory in SARS-CoV-2:

Of the studies included, 6 investigated the anti-inflammatory effects of heparin and heparin forms in relation to SARS-CoV-2, studies included one *in vitro* study, one clinical trial, three retrospective cohort studies and two prospective observational studies, as seen in *Table 11*.

Table 11: Effect of low molecular weight heparins and mimetics on clinical, laboratory, coagulation and anti-inflammatory markers, adapted from (Buijsers, et al., 2020) (Gonzalez-Ochoa, et al., 2020) (Shi, et al., 2020) and (Yormaz, et al., 2020).

CS: retrospective cohort study, RCT: randomised control trial, PCS: prospective cohort study

↓: decreased with treatment ↑: increased with treatment, none: no change seen, -: no data

Author (reference)	Design	Patients (n)	Heparin form	Mode of treatment		Clinical parameters				Laboratory findings	Coagulation parameters	Anti-inflammatory markers			
				P/T	S/C	Need of stay	Duration of stay	Mechanical ventilation	Mortality	Lymphocytes	D-dimer	CRP	Cytokine	Creatinine levels	FDP
(Shi, et al., 2020)	RCS	42	LMWH	T	C	-	None	-	-	↑	↓	-	↓IL-6	-	↓
(Gonzalez-Ochoa, et al., 2020)	RCT	243	Sulodexide	T	S	↓	-	↓	↓	-	↓	↓	-	-	-
(Buijsers, et al., 2020)	RCS	48	Dalteparin	P	C	-	↓	-	↓	-	↑	-	-	-	-
(Yormaz, et al., 2020)	PCS	96	LMWH	T	C	-	↓	-	None	↑	↓	↓	None	-	↓

To establish the basis of heparin as an anti-inflammatory treatment in the case of SARS-CoV-2, molecular dynamic simulations of LMWH with the full length IFNγ and IL-6 were carried out by, Litov, *et al.*, 2020. Interferon-γ signalling pathway coordinates several biological responses, primarily involved in host defence against intracellular pathogens and immune surveillance but also in the establishment of adaptive immunity and in the regulation of inflammation, apoptosis, and

cell cycle (Ivashkiv, 2018). In summary, IFN-γ strongly promotes innate immune and inflammatory responses, important for local differentiation of monocytes into dendritic cell and macrophages (Goldszmid *et al*., 2012), important for local antiviral responses (Weizman *et al*., 2017) and are an important in vaccine induced memory via memory T-cell derived IFNγ (Soudja, Ruiz, Marie and Lauvau, 2012). Interferon-γ is secreted predominantly by activated lymphocytes such as CD4 T helper type 1 (Th1) cells and CD8 cytotoxic T cells, γδ T cells, and natural killer cells, and to a lesser extent natural killer T cells, B cells and professional antigen presenting cells (Castro *et al*., 2018). A large body of evidence documents the proinflammatory nature of IFNγ, which has allowed IFNγ to function as a marker of inflammation and autoimmune disease (Zhang, 2007). Similarly, IL-6 has pleiotropic activity, it is promptly produced in response to infections and tissue injuries, contributes to the host defence by stimulation of acute phases responses, haematopoiesis, and immune reactions (Scheller, Garbers and Rose-John, 2014). Interleukin-6 induces the synthesis of acute phase proteins such as CRP, serum amyloid A, fibrinogen and hepcidin in hepatocytes, whereas it inhibits production of albumin (Velazquez-Salinas, Verdugo-Rodriguez, Rodriguez and Borca, 2019). In addition, IL-6 also has a role in the acquired immune response by stimulation of antibody production and effector T-cell development (Dienz *et al*., 2009). Although, IL-6 typically contributes to host defence, dysregulation or excessive synthesis of IL-6 and other cytokines leads to an acute systemic inflammatory response, which can result to an acute severe systemic inflammatory response cytokine storm (Copaescu *et al*., 2020). The pathogenesis of SARS-CoV-2 involves a potent inflammatory response, data has demonstrated that IL-6 kinetics are highly related to disease severity (Zhang *et al*., 2020) (Han *et al*., 2020).

Given the roles in innate immunity and inflammation, the ongoing research into the different prognostic markers of disease progression, IL-6 and IFNγ are potential targets for SARS-CoV-2 therapy. Litov, *et al*., 2020, demonstrated that LMWH binds to the C-termini of IFNγ with high affinity to form a very stable complex. Binding of two or more LMWHs results in a complex with an overall negative net charge, this acts to prevent the first necessary step in the IFNγ transduction pathway by inhibiting further interaction of IFNγ with the extracellular region of the IFNGR1. Further simulations investigating the binding activity of LMWH to IL-6 show revealed that LMWH interacts with IL-6 in two ways, one of which results in the formation of a stable IL-6-LMWH, heparin blocks the site I of Il-6 and inhibits binding with IL-6Rα. The second involves LMWH binding to the IL-6/IL-6Rα complex blocking the binding site II of IL-6 and preventing gp130 receptor binding. To study the inhibition of IFNγ and IL-6 *in vitro* a series of assays were carried out. The antiproliferative activity of IFNγ was used as the basis to study the effect of a hexasaccharide LMWH, by measuring the production of the inductive enzyme indoleamine-2,3-dioxygenase (IDO) in the presence of different LMWH concentrations. LMWH was found to inhibit the antiproliferative activity of IFNγ in a concentration dependent manner, with an IC_{50} of

approximately 35IUml^{-1}. A LMWH concentration of 150IUml^{-1} reduced the induction of IDO by up to 80% whilst remaining nontoxic to cells. Further investigation studying the translocation of phosphorylated STAT1 in the nucleus revealed that LMWH abolishes the activation of the JAK/STAT1α pathway, showing full inhibition at 150Uml^{-1}.

Shi, *et al*., 2020 conducted a retrospective cohort study of 42 patients, the control arm consisted of 21 patients who did not receive LMWH treatment and the remaining 21 in the treatment group who did receive LMWH treatment during hospitalisation. The most significant differences seen between the two groups included the changes in lymphocyte percentage before and after treatment. With those in the treatment group benefiting from an increase in lymphocyte percentage, 11·10±9·50 compared to 3·08±9·66 (p=0.011) in the control group. As expected, coagulation markers, D-Dimer and fibrinogen degradation products (FDP) in the LMWH group before and after treatment were significantly different in comparison to the control group (-2·85±3·90, -0·05±0.85, *p*=0·002; -9·05±13·14, -1·78±3·15, *p*=0·035). Most strikingly, the IL-6 levels of the LMWH treatment group were significantly reduced following treatment 47·47±58·86, compared to 15·76±25·71 prior to treatment, *p*=0·006. Not only this, but the changes in IL-6 were significantly different from those observed in the control group (-32·46±65·97, 14·96±151·09, *p*=0·031).

A similar prospective cohort study was carried out by, Yormaz, *et al*., 2020, consisting of 96 patients, separated into two groups according to D-dimer levels (D-dimer > 750 ng.ml^{-1}) and prothrombin time (<12 seconds) outcomes due to mortality. Both groups consisted of 48 patients, one of which received LMWH and the other did not, patient outcomes were followed for 7 days. LMWH treatment was found to have a positive impact on the number of days to converting the virus to a negative outcome, LMWH: 5.2 days and control group: 7.6 days, p=<0.001) and duration of hospital stay, LMWH: 7.2 days and control group: 9.6 days. Lymphocyte count of the LMWH treatment group were significantly elevated following treatment, 1.39 ± 0.40k.µl^{-1} compared to the control group, 1.02 ± 0.03k.µl l^{-1} (p=<0.001), consistent with Shi, *et al*., 2020. Similarly, D-dimer, fibrin degradation products and CRP were significantly decreased following treatment, with p values of <0.001 for all markers, as seen in Shi, *et al*., 2020. Although promising, CRP is not a specific marker of viral infection progression, but is a non-specific marker of inflammation, as transcriptional induction of the CRP gene mainly occurs in the liver in response to increased levels of inflammatory cytokines, particularly, IL-6 (Boras *et al*., 2014). For this reason, CRP values cannot be used alone to compare the anti-inflammatory properties of a treatment, but can be used to supplement other measurements. However, there were no significant differences observed in the creatine kinase isoenzyme B (CK-MB) levels (p=0.663) or troponin-I levels (p=0.089), between the two groups.

A prospective randomised placebo-controlled trial with a parallel group was carried out by, Gonzalez-Ochoa, *et al*., 2020, to evaluate the effect of sulodexide on the clinical outcome of consecutive patients with early clinical stages of SARS-COV-2. Of the 312 patients randomised for group allocation, 69 either tested negative or lacked sufficient data, resulting in the enrolment of a total of 243 patients into the trial. As a result, there were 124 patients in the sulodexide group and 119 in the placebo group. During the 21 days of follow up, 57 patients required hospital care, 17.7% of the sulodexide group and 29.4% of the placebo group. Despite this, sulodexide appeared to have little effect on duration of hospital stay, the sulodexide group had a mean of $6\cdot2 \pm 4\cdot1$ days compared to $7\cdot8 \pm 4\cdot5$ days in the placebo group, however, this was not statistically significant p=0.21. There were 87 out of 243 patients that developed respiratory symptoms that necessitated oxygen support, 35.8% of the sulodexide compared to 42% of the control group. This decrease in need for mechanical ventilation was significantly different, p=0.05, not only did sulodexide decrease the need for oxygen support but significantly reduced the length of treatment for those who did, $9 \pm 7\cdot2$ days in the sulodexide group compared to $11\cdot5 \pm 9\cdot6$ in the placebo group; p=0·02. Sulodexide did decrease the overall mortality rate, 4.1% in the sulodexide group compared to 5.8% of the placebo, however, this effect was not statistically significant p=0·19. Sulodexide appears to decrease the risk of thrombus formation, as levels at week 2 were significantly ($p<0\cdot01$) increased in the placebo group, $897\cdot7 \pm 1215\cdot36$ng/ml compared to the treatment group, $464\cdot75 \pm 629\cdot81$. Furthermore, $21\cdot7\%$ patients in the sulodexide group showed a D-dimer value >500 ng/dl compared to $47\cdot05\%$ in the placebo group $p<0\cdot01$. Similarly, sulodexide decreases C-reactive protein concentration at week 2, with $12\cdot55 \pm 10\cdot2$ mg/dL in the sulodexide group compared to $17\cdot81 \pm 11\cdot56$ mg/dL in the placebo group, $p<0\cdot01$.

A retrospective cohort study by, Buijsers, *et al*., 2020, investigating the inhibition of heparanase using dalteparin, found that this could be a potential treatment for moderately diseased SARS-COV-2 patients, not those requiring intensive care. There were 48 patients enrolled onto the study, with 14 in the ICU and 34 in SARS-COV-2 clinical wards, this group was further divided into a prophylactic LMWH treatment group (n=17), those receiving alternative anticoagulation (n=8) and patients who had not had any medical intervention prior to the study, the control group (n=9). Increased heparanase (HPSE) activity is known to lead to dysfunction in the endothelial barrier, which may contribute to the development of ARDS. It was found that SARS-COV-2 patients had increased HPSE and IL-6 levels patients compared to healthy controls. Furthermore, increased HPSE activity was associated with severity of SARS-COV-2, with HPSE levels of the ICU group significantly higher than those in the non-ICU group. Additionally, increased LDH and serum creatinine values were also associated with higher HPSE activity levels. Prophylactic LMWH, in this case dalteparin, in non-ICU patients resulted in significantly lower plasma HPSE activity compared to the control group. *In vitro* studies confirmed these findings, using an ELISA it was

demonstrated that inhibition of HPSE was dose dependent at concentrations between $0.0025U.ml^{-1}$ and $0.05U.ml^{-1}$ and full inhibition starting from $0.25U.ml^{-1}$. This suggests that dalteparin was acting prophylactically, as one single dose of dalteparin of 5000U, results in an estimated concentration of approximately $0.37U.ml^{-1}$ *in vivo*. However, no statistical differences were found between the IL-6 levels of non-ICU patients in the control or treatment group. Prophylactic dalteparin treatment appeared to have no significant impact on mortality, p=0.6012 and significantly increased the duration of stay, p=0.0443.

Studies indicate that 20-30% of SARS-CoV-2 patients will present with or develop delirium during their hospitalisation, in severe cases this increases to 60-70% (O'Hanlon and Inouye, 2020). The mechanisms of this are likely multifactorial including direct neurological invasion, or indirectly through hypoxia, fever, metabolic derangements, or inflammation due to cytokine storms. C reactive protein is an acute phase protein of hepatic origin that increases following IL-6 secretion by macrophages and T cells (Sproston and Ashworth, 2018). A smaller prospective cohort study by D'Ardes, *et al*., 2020, of 56 patients, investigating the relationship between LMWH and delirium. It was found that enoxaparin treatment significantly reduced the risk of delirium, p=0.004 and reduced the levels of C reactive protein compared to the control group, as in Gonzalez-Ochoa, *et al*., 2020 and Yormaz, *et al*., 2020. In addition, enoxaparin treatment reduces length of stay significantly, 27.0 ± 14.7 in the untreated group compared to 21.5 ± 10.1; $p = 0.04$. This data cannot be concretely linked to inflammation as delirium cannot be linked to one single factor, however, it is promising that the use of LMWH reduces the risk of delirium whilst reducing the levels of the non-specific inflammation marker CRP.

Coagulation as a parameter of heparin treatment:

A retrospective cohort study consisting of 2075 patients, investigating the relationship between heparin and survival in patients was carried out by, Ayerbe, *et al*., 2020. Data were extracted comparing the incidence of mortality in patients treated with heparin compared to those without. Findings showed that heparin significantly reduced the incidence of mortality, 13.96%, compared to 15.44% in the control group. A similar retrospective cohort study, Tang, *et al*., 2020 found that mortality was not affected by the use of heparin in patients with a SIC (sepsis induced coagulopathy) score of <4 or D-Dimer <6-fold of upper limit of normal. 28-day mortality of the control group was 29.7% compared to 30.3 in the heparin treatment group, however, in patients with a SIC score ≥ 4, a significant (p=0.029) reduction in mortality was seen, with 40% in the heparin group compared to 60% of the control. When taking into account the D-dimer results, the mortality in the heparin group remained fairly consistent, whereas in non-users the mortality rose proportionally with increasing D-dimer. When D-dimer levels exceeded 3.0µgml^{-1} heparin treatment reduced mortality by approximately 20%, with the incidence of mortality 32.8% and 52.8% in the heparin and control group respectively.

Type of heparin:

The efficacy of unfractionated heparin and the low molecular weight heparin, enoxaparin was evaluated by, Pawlowski, *et al*., 2020. In this retrospective cohort study, a dataset of 671 hospitalised patients was compared to determine the effects on mortality, ICU admission, lengths of stay and thrombotic events. Patients in the heparin only group had a higher risk of mortality, 16% compared to 7.7% in the enoxaparin group and a higher risk of admission to the ICU, 34% compared to 19% in the enoxaparin group. Furthermore, enoxaparin treatment was associated with a significantly reduced duration of stay in the hospital and ICU, with the heparin only group having a mean hospital stay of 6.3 days, 1.6 of which were ICU free compared to a mean hospital stay of 4.9 days, 2.5 of which were ICU free. There was a greater incidence of thrombotic complications in the heparin cohort, 10.1% compared to 0.8% in the enoxaparin group, in particular deep vein thrombosis, had an incidence of 2.9% in the heparin group compared to 0.2% in the enoxaparin group. Consistent with this, (Arslan, *et al*., 2020), found in a retrospective cohort study of 413 patients that enoxaparin reduced the length of stay significantly, with an average length of stay for treated patients of 8.2±3.6 days and 10.2±4.1 for the untreated group, p<0.001. Similarly, it was found that enoxaparin treatment significantly decreased the ICU admission rate of the patients, only 6 patients were transferred to the ICU in this study, all of which were in the untreated group.

Correspondingly, the efficacy of LMWH was evaluated in a retrospective cohort study of 525 patients was carried out by, Shen, *et al*., 2021. Patients were divided into two groups, LMWH

treatment group (n=120) and non-LMWH group (n=405), it was found that compared with the LMWH group, the non-LMWH had a lower unadjusted in-hospital mortality rate, 11.1% compared to 21.7%. Despite this, the use of LMWH was associated with a lower adjusted mortality risk compared to the non-LMWH group, OR: 0.18. A greater survival benefit was observed among severely and critically ill patients as well as the elderly (>65) and patients with IL-6 > 10times upper limit level and D-dimer > 5 times upper limit level.

To determine whether the heparin mimetic fondaparinux could be an effective treatment in the case of SARS-COV-2 a retrospective cohort study was undertaken by, Russo, *et al*., 2020 to compare the safety, effectiveness and impact on clinical prognosis of fondaparinux and enoxaparin. The study consisted of 120 patients, of which 46 were administered fondaparinux and the remaining 74 taking enoxaparin. In terms of mortality there was no significant difference between the two groups, the incidence of mortality in the enoxaparin group was 9.5% compared to 10.9% in the fondaparinux group, although the risk was slightly higher in the fondaparinux group it is not statistically significant, p=0.99. However, fondaparinux appeared to have a greater net clinical benefit, reducing the risk of developing ARDS, 15.2% compared to 18.9% for the enoxaparin cohort, similarly, fondaparinux reduced the incidence of VTE and bleeding events. Through this data it was found that fondaparinux had a net clinical benefit over enoxaparin equal to +4.6.

Prophylactic versus therapeutic therapy:
The assessment of pre-emptive therapeutic dose anticoagulation compared to therapeutic treatment was carried out in a retrospective cohort study by Motta, *et al*., 2020. It was found that prophylactic heparin treatment was associated with a lower incidence of mortality 14.4% compared to 38.7% in the therapeutic group. In contrast, Trinh, *et al*., 2020, found in a retrospective cohort study evaluating prophylactic compared to therapeutic treatment in mechanically ventilated patients, that therapeutic treatment is associated with decreased mortality. The records of 245 patients were reviewed with the view to compare mortality and morbidity, it was found that patients who received therapeutic doses of heparin and enoxaparin or both, had a survival advantage of 57% compared to 25% in the prophylactic group. Patients in the therapeutic group had a longer length of stay in the ICU, 18 days, compared to the prophylactic group, 11 days. In disagreement with both, Motta, *et al*., 2020 and Trinh, *et al*., 2020, Bolzetta, *et al*., 2020 found in a retrospective cohort study focussing on the older generation (60 years and older) that there is no significant difference in survival rate of patients treated therapeutically or prophylactically. The only significant difference between the cohorts was serum creatine levels, 1.61 ± 1.11 in prophylactic doses compared to 1.22 ± 0.52 in therapeutic doses; p value $= 0.04$.

A further retrospective study comprised of 56 patients aimed to compare the incidence of thromboembolic events in adult patients with SARS-COV-2 treated with UFH compared to

prophylactic dose anticoagulation (Li, *et al*., 2020). There was no difference in the composite of thromboembolic events between the two groups, 17.9% compared to 3.6%, p=0.19. However, patients in the UFH group were more likely to receive renal replacement therapy, 35.7% compared to 3.6%, p=0.005, more mechanical ventilation, 75% compared to 25%, p<0.005 and required longer durations of mechanical ventilation 13.7 days±7.4 compared to 1.7 days±3.8, P < 0.005. Continuous infusion of UFH also increased the incidence of minor bleeding, 37.5% compared to the control group, 0% and needed significantly more units of packed red blood cell transfusion, 0.8 units ± 1.6, compared to 0 for the control group. there was, however, no significant difference in the incidence of major bleeding events, p=0.49. UFH treatment did appear to stabilise D-dimer levels, with a modest decrease seen in concentrations from day 1 to day 7 in the control group, 1412ng.ml^{-1}, compared to the significant increase seen in the control group 10345ng.ml^{-1}. Like, Bolzetta, *et al*., 2020, it was found that there was no significant difference in the incidence of mortality between prophylactic, 32.1% and therapeutic UFH, 17.9%, p=0.36.

Time of dose was further investigated for low molecular weight heparin, in particular enoxaparin by Paolisso, *et al*., 2020. The study consisted of 450 laboratory-confirmed SARS-COV-2 patients, of which 361 received standard prophylaxis treatment and 89 received therapeutic enoxaparin dosage for seven days. Therapeutic LMWH was associated with a lower mortality compared to standard prophylaxis, 18.8% and 5.8% respectively (p=0.02) and with reduced length of hospital stay, 8 days compared to 8 in the prophylactic group, although this was not statistically significant. However, prophylactic treatment reduced risk of major bleeding events, 0.6% in the prophylactic group compared to 2.2% in the therapeutic group. However, an observational study by Albani, *et al*., 2020 consisting of 1403 patients found that enoxaparin thromboprophylaxis was associated with reduced risk of ICU admission and lower in hospital mortality but unlike as found in Paolisso, *et al*., 2020 is associated with an increased duration of stay. With regards to thrombotic and haemorrhagic events, the enoxaparin group was divided into two, prophylactic and therapeutic, increased incidence of both was seen in the therapeutic group, 28% and 3.2% respectively. Prophylaxis appeared to be protective against thrombotic events as the difference is statistically significant, with an incidence of 2.5%, p<0.001, however, the same effect was not seen in the case of haemorrhagic events,1.2%, p=0.12.

A randomised, open label, phase II study carried out by Bertoldi Lemos, *et al*., 2020 evaluated the efficacy of both therapeutic enoxaparin and the standard anticoagulant thromboprophylaxis in patients with respiratory failure requiring ventilation. The study consisted of 20 patients, 10 of which were assigned to therapeutic enoxaparin and ten to prophylactic anticoagulation. The therapeutic group experienced a significant increase in the PaO$_2$/FiO$_2$ ratio over time, p=0.0004, however this improvement was not seen in the prophylactic group. Similarly, the ratio for successful liberation from mechanical ventilation was increased in the therapeutic group, 4 compared to the

prophylactic group, similarly, the therapeutic group had more ventilator free days, 15 days, compared to the prophylactic group, which had none. The D-dimer levels of the therapeutic group significantly decreased over time to 1469μg.l^{-1} p=0.009, yet significantly increased in the prophylactic group to 4878μg.L^{-1}, p=0.004. There was no statistical significance of the difference in the mortality rate and ICU free days between the two groups.

To determine whether prophylactic anticoagulant treatment within 24 hours of admission is associated with decreased risk of death in SARS-CoV-2 patients, an observational cohort study was carried out by Rentsch, *et al*., 2020. The study consisted of 4297 patients hospitalised with SARS-COV-2, 84.4% (n=3627) received prophylactic anticoagulative within 24 hours of admission, of which more than 99% (n=3600) received subcutaneous enoxaparin or heparin. It was found that risk of death within 30 days of hospital admission was significantly reduced in the prophylactic group, with a 27% decrease in risk of death compared to those not receiving prophylactic anticoagulation. Similarly, decreases were seen in the treatment groups inpatient mortality and initiation of therapeutic anticoagulation. In post-hoc analyses revealed that the effect of prophylactic anticoagulation was similar whether patients received subcutaneous enoxaparin with a hazard ratio of 0.78 or heparin, hazard ratio: 0.73. A further study by, Canoglu & Saylan, 2020, determined the efficacy of prophylactic compared to therapeutic treatments. The study aimed to investigate the relationship between coagulation parameters and the dose of enoxaparin, mortality and ICU admission in hospitalised patients with severe SARS-COV-2 pneumonia. The retrospective cohort study consisted of 154 patients, 98 of which were treated with a prophylactic dose of enoxaparin and 56 treated with a therapeutic dose. Significantly, 44.9% of the prophylactic dose group died, compared to the therapeutic dose, 17.9%, p=0.001. Mortality was 6.4-fold higher in the prophylactic group compared to the therapeutic enoxaparin dose users, p=<0.001.

The impact of anticoagulation treatment on the time to death in SARS-COV-2 was investigated by Ionescu, *et al*., 2020 in a prospective cohort study of 127 patients. Of which 67 received therapeutic anticoagulation, 47 received prophylactic treatment and 13 received no anticoagulant treatment at all. Therapeutic anticoagulant treatment was defined as, UFH as an intravenous infusion with documented activated partial thromboplastin time (aPTT) in the AC range (≥45 seconds) or subcutaneous enoxaparin at doses of 1 mg/kg twice daily or 1.5 mg/kg once daily and prophylactic anticoagulant treatment was defined as, subcutaneous injection of UFH at doses of 5,000 units twice or three daily, or subcutaneous enoxaparin injection at doses of 30 to 40 mg once daily. Bleeding rates were extremely similar between the two groups, p=0.877, 19% and 18% for the therapeutic and prophylactic group respectively. Significantly, median time to death was longer in cases with higher doses of anticoagulation treatment, 11 days for therapeutic coagulation, 8 days for prophylactic treatment compared to 4 days for those who did not receive any anticoagulant treatment at all, p<0.001.

A larger, retrospective cohort study of 3480 patients was carried out again by, Ionescu, *et al*., 2020b, to further investigate the dose and duration dependent delay in death of SARS-COV-2 patients. Most patients received prophylactic treatment, 60.9% and almost one third received therapeutic treatment for three days or longer, 28.7% and 10.4% received no treatment at all. The primary prophylactic anticoagulants were enoxaparin (54.5%), UFH (33%) and fondaparinux (0.3%) whereas therapeutic treatment consisted of 42.5% enoxaparin, 29.6% UFH and 0.6% fondaparinux, with the rest receiving oral anticoagulation. In-hospital mortality was increased in the therapeutic group as in the previous study, 23.6% compared to 10.8% and 11.4% or the prophylactic and no treatment group respectively. However, therapeutic treatment was associated with longer survival time, 30 days compared to 25 days for prophylactic anticoagulation treatment. At day 25, the survival probability was higher in the therapeutic group, 57.5% compared to 50.7% for the therapeutic group. Therapeutic treatment was also associated with an increase in survival probabilities 25 days following admission in both the ICU and non-ICU patients, 56.3% compared to 22% and 78% compared to 65.7%. However, prophylactic treatment had a protective effect against major bleeding events, with an incidence of 2.3% compared to 8.1% in the therapeutic group and 5.5% in the no treatment group. This research is consistent with the prior study, anticoagulant treatment reduces the risk of death in a dose dependent manner, a multivariate model showed that compared to no anticoagulant treatment, prophylactic treatment was associated with a 65% decrease in risk of death and therapeutic treatment reduced the risk by 86%.

Dosing strategy:

A retrospective cohort study, by Jonmaker, *et al*., 2020, investigated the dosing strategy of thromboprophylaxis in critically ill SARS-COV-2 patients to determine if whether higher doses are associated with a lower mortality rate. This retrospective study, 152 critically ill patients were divided into three groups, low: 2500–4500IU tinzaparin or 2500–5000IU dalteparin, medium > 4500IU but < 175IU/kilogram of body weight tinzaparin or > 5000IU but < 200IU/kg of body weight dalteparin, and high dose ≥ 175IU/kg of body weight tinzaparin or ≥ 200IU/kg of body weight dalteparin. Patients receiving high-dose prophylaxis had significantly lower mortality, 13.5%, compared to medium dose, 25% and low dose, 38.8% (p=0.02). High dose thromboprophylaxis also had a significantly reduced hazard ratio, 0.33, compared to medium dose, 0.88. the risk of death did not differ between groups until 7 days following admission, after which the proportion of deaths increased in the low-dose thromboprophylaxis group compared to the other groups. The median number of ICU free days alive were, 0 with low dose, 11 with medium dose and 18 with high-dose thromboprophylaxis, p=0.07. Similarly, the proportion of thromboembolic events were significantly decreased with high dose thromboprophylaxis (p=0.07), 2.7% compared with 18.8% and 17.9% with medium and high dose respectively. Furthermore, high dose thromboprophylaxis significantly decreased the level of D-Dimer, p=0.002, creatinine, p<0.001 and

CRP, p=0.01. In contrast, high dose thromboprophylaxis increased the level of haemoglobin, p=0.01 and platelet count, p=0.003, compared to low and medium dose. In contrast, Rivera-Izquierdo, *et al*., 2020, found in a retrospective cohort study of 238 patients found that increasing dose increased the risk of mortality. Using the low molecular weight heparin, bemiparin sodium at increasing concentrations, 2500-3500U, 5000-7000U and 7500-10,000U resulted in hazard ratios of 0.76, 1.24 and 1.40 respectively.

Similarly, Martinelli, *et al*., 2021 carried out an observational cohort study with 278 hospitalised patients to determine if high dose enoxaparin was more effective as a treatment than a standard dose. As in Jonmaker, *et al.,* 2020, high dose enoxaparin was associated with a lower risk of death with a mortality rate of 0.25 compared to almost double that in the standard treatment group, 0.56. Likewise, high dose enoxaparin treatment was associated with a lower risk of transfer to the ICU resulting in the observation of a reduction of 60% of mortality and clinical deterioration in this group. However, 3% of patients receiving high dosage enoxaparin had non-fatal major bleeding events, whilst the standard dose cohort had none, despite this the high dosage group had a 50% reduction of venous thromboembolism compared to standard dosage prophylaxis, which is consistent with findings by Jonmaker, *et al.,* 2020. In contrast, Rivera-Izquierdo, *et al.,* 2020, found in a retrospective cohort study of 238 patients found that increasing dose increased the risk of mortality. Using the low molecular weight heparin, bemiparin sodium at increasing concentrations, 2500-3500U, 5000-7000U and 7500-10,000U resulted in mortality hazard ratios of 0.76, 1.24 and 1.40, respectively.

Heparin compared to other anticoagulants:
To evaluate the effects of heparin treatment compared to other anticoagulant treatments, a prospective cohort study was carried out by, Billet, *et al*., 2020, to investigate the relationship of both the type and intensity of anticoagulants to mortality. A total of 3,625 patients were enrolled in the study, of which 2450 had complete data sets. It was found that prophylactic enoxaparin treatment was the most effective heparin treatment with an odds ratio of 0.49 (p=0.001), compared to, 0.83 for enoxaparin full therapy, 0.79 for UFH prophylaxis twice daily, 1.04 for UFH prophylaxis thrice daily and 0.97 UFH full therapy. All heparin forms except UFH prophylaxis thrice daily were protective against mortality compared to no anticoagulation treatment at all (OR=1), however, UFH prophylaxis thrice daily was associated with an increased risk of mortality. Although heparin treatments are effective, it was found that prophylactic and therapeutic treatment using apixaban was more effective, OR 0.46 and 0.57 respectively.

Similarly, a prospective cohort study consisting of 844 patients compared the efficacy of pre-hospitalisation oral anticoagulation (OAC) with therapeutic heparin treatment in mortality among patients with SARS-COV-2 Schiavone, *et al*., 2021. It was found that heparin was associated with

a better chance of survival to hospital discharge (OR 0.60 [0.38–0.94], p < 0.001), in particular when compared to patients with acute hypoxemic respiratory failure (AHRF). Furthermore, the highest mortality rate was seen in AHRF patients when heparin was not administered. When taking into account D-dimer values, the effects were variable, at < 1µg.ml-1 there was no benefit associated with any of the treatments, whereas at levels 1- < 3µg.ml-1 treatment with prophylactic apixaban and enoxaparin and therapeutic apixaban was associated with a significant decrease in mortality. At increasing D-dimer levels, > 10µg.ml-1, the same pattern was seen, effective treatment using prophylactic and therapeutic apixaban and prophylactic enoxaparin with no discernible beneficial effects of UFH or full enoxaparin therapy. These findings disagree with Ayerbe, *et al.,* 2020, where it was found that UFH reduced the incidence of mortality by 20% when D-dimer levels exceeded 3.0µgml-1.

Table 12: Effect of different heparin forms in a clinical setting comparing dosage, clinical markers and laboratory characteristics, length of stay, admission to ICU and mortality. *RCS: retrospective cohort study, RCT: randomised control trial, OS: observational study, E: enoxaparin, F: fondaparinux, UFH: unfractionated heparin, T: therapeutic P: prophylactic -: no data recorded*

Author (reference)	Design	Patients (n)	Type of heparin	T/P	Dose	HR (Mortality)	Mortality (%)	Length of stay (Days)	Mechanical Ventilation Free days (Days)	PaO₂/FiO₂	ICU Free days (Days)	ICU Admission rate (%)	ICU Duration (Days)	Thromboembolic events %	FDP (mg.l⁻¹)	CRP (mg.l⁻¹)	Haemoglobin (g.l⁻¹)	Creatinine (mg.l⁻¹)	Platelet count (µ/l)	INR	D-dimer (µg.ml⁻¹)
(Jonmaker, et al., 2020)	RCS	152	T/D	P	Low: 2500-4500U/ 2500–5000IU	1	38.8				0			14.9	6.4	335	94	100	203	1.1	
					Medium: > 4500 IU/ > 5000 IU	0.88	25	-	-	-	11	-	-	18.8	2.8	290	102	78	257	1.1	-
					High: ≥ 175 IU/kg ≥ 200 IU/ kg of body weight	0.33	13.5				18			2.7	1.7	229	107	66	314	1	
(Pawlowski, et al., 2020)	RCS	671	UFH	T	-	-	16	6.3	-	-	1.6	29	-	10.1	-	-	-	-	-	-	-
			E				7.7	4.9			2.5	26		0.8							
(Ayerbe, et al., 2020)	RCS	2075	UFH	T	-	-	13.96	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Motta, et al., 2020)	RCS	374	UFH	P	5000 units given SC/8hr	-	14.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			E		30/40 mg SC daily																
			UFH	T	IV titrated to aPTT between 70-110 sec	-	38.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			E		1 mg/kg SC twice daily/ 1.5 mg/kg SC daily																
(Trinh, et al., 2020)	RCS	245	E	P	40mg twice daily (GFR > 30ml/min) or 40mg daily (GFR ≤ 30ml/min)	-	34.2	15.7±8.9	-	-	-	-	18	26.5	-	-	-	-	-	-	-
			H		5000 units SC 2/3 times daily																
			E	T	1mg/kg twice daily (GFR > 30ml/min)/ once daily (GFR ≤ 30ml/min)	-	53	23.3±7.7	-	-	-	-	11	45.4	-	-	-	-	-	-	-
			H		15u/kg/hr																
(Tang, et al., 2020)	RCS	449	E	T	40-60 mg/d	-	30.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			UFH		10 000-15 000U/d																
(Paolisso, et al., 2020)	RCS	450	E	P	40-60mg/day		20.8	10			15.8			0.6		5.9	13.2	0.92	194		
				T	40–60 mg twice daily		4.5	8	-	-	14.6	-	-	2.2	-	6.3	13.8	0.87	193	-	-
(Bertoldi Lemos, et al., 2020)	RCT	20	E	P	40mg OD/ 40mg BID		50	30	0	195	0			-		-	-	-	-	-	4.878
			UFH		SC 5000-7500IU TID																
			E	T	SC 0.75-1mg.kg⁻¹ BID		20	31	15	261	12	-	-	-	-	-	-	-	-	-	1.469
(Rivera-Izquierdo, et al., 2020)	RCS	238	BS	T	2500-3500U	0.76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
					5000-7000U	1.24															
					7500-10000U	1.43															
(Albani, et al., 2020)	RCS	1403	E	T	40-80mg	-	25.5	9	-	245	-	10.4	-	16	-	119.7	-	-	190	-	-
				P	40-80mg	-	-	-	-	-	-	-	-	2.5	-	-	-	-	-	-	-
(Martinelli, et al., 2021)	OS	278	E	P	Standard: 40mg/day	-	13.9	-	-	-	-	-	-	13.2	-	-	-	-	-	-	-
					High: 1mg/kg ICU, 0.7mg/kg high intensity of care wards, 1mg/kg	-	9.4	-	-	-	-	-	-	15.7	-	-	-	-	-	-	-

Discussion:

This scoping review presents six main findings, first, that heparin can competitively inhibit HS binding of SARS-CoV-2 to target host cells, independent of the angiotensin 2 receptor. Furthermore, this inhibition extends to other heparin forms such as low molecular weight heparins, modified heparins, most significantly O-sulphated heparin forms and heparin mimetics such as fondaparinux and pixatimod. Secondly, low molecular weight heparin or similar mimetics appear to be the most promising treatment candidates *in vivo* and *in vitro*. Thirdly, sulphated polysaccharides, particularly from marine plant sources have potential to be effective treatments with limited cytotoxicity for SARS-CoV-2. Fourth, *in vivo*, treatment of SARS-COV-2 with heparin is associated with a significantly reduced mortality risk, when compared to other standard treatments such as hydroxychloroquine and remdesivir. Fifth, heparin treatment can act to modulate inflammatory responses *in vivo* with positive effects, yet more research is required. Finally, that heparin can be used prophylactically and therapeutically with benefits in both cases, but no clear conclusion as to the best treatment strategy.

Heparin has been found to bind to SARS-CoV-2 *in vitro*, to induce conformational secondary structure changes. Binding has been shown to occur with varying protein constructs, the full-length receptor binding domain, the full-length monomeric spike protein and the trimeric spike protein and S1 and S2 subunits with differing affinities. Agreement with regards to the affinity of binding interactions has not yet been found in the literature qualitatively. However, the most potent binding appears to be between heparin and the full-length trimeric spike protein, with the polybasic furin cleavage site revealed as a potential binding site. In addition, heparin has been shown to inhibit plaque formation in Vero cells, up to 80%, with a similar effect seen in further studies with RT4 cells. Both of which displayed that heparin could inhibit binding to cells at prophylactic and therapeutic concentrations. Further studies show that heparin can be used to inhibit SARS-CoV-2 host cell binding in other cell lines, primary human bronchial epithelial cells, human lung epithelial Calu-3 cells and HEK293T and against other clinical strains, SARS-CoV-2, USA-WA1/2020. Analysis of studies evaluating heparin oligosaccharide chain length revealed that there appears to be no clear relationship between chain length and inhibitory activity, with tetrasaccharides to decasaccharides displaying inhibitory activity to varying degrees in the number of studies shown. However, the role of sulphation seems to show more consistent results, with O-sulphation, particularly, 2-O sulphation, but importance has noted for 3-O and 6-O sulphation respectively, yet the evidence is much less consistent. N-sulphation appears to have a lesser role in inhibition of SARS-CoV-2 binding, with reduction in activity seen in N-desulphated forms, yet the difference is less significant when compared to de-O-sulphated heparins.

Low molecular weight heparin is often used clinically with advantages over unfractionated heparin with regards to bioavailability, half-life and more simplified dosage strategies (Solari & Varacallo,

2020) (Merli & Groce, 2010). A range of LMWH have been found to have inhibitory action against SARS-CoV-2 *in vitro*, however the degree of activity varies, and findings are not yet consistent. Enoxaparin has been found to be the most promising candidate with inhibitory activity in a number of studies, however, only one study finds its activity comparable to heparin and another study found that it has no inhibitory effect up to 1000µg.ml^{-1}. In contrast, tinzaparin, dalteparin and nadroparin had less research data available, with only one study included on their inhibitory effect *in vivo*. It was found that tinzaparin was the most effective at inhibiting viral cell binding at 100IU, whereas dalteparin was more effective at inhibiting infection transmission at, 50IU. Three heparin mimetics were included in this scoping review: fondaparinux, pixatimod and SPGG. The latter has only one study associated with it; however, it was found to successfully inhibit cell-to-cell fusion in both human HEK293T and CHO-K1 cell lines more effectively than fondaparinux. Pixatimod was also identified as a potential treatment for SARS-CoV-2, with a series of assays determining that it effects the secondary structure of recombinant spike protein receptor binding domain. Pixatimod was also found to destabilise mammalian expressed SARS-CoV-2-RBD more significantly than heparin. However, inhibition assays revealed that heparin was a more potent inhibitor of EcS1-RBD protein to Vero cells than pixatimod. With regards to fondaparinux, the outcomes of research vary, with (Hao, *et al*., 2020) finding that it bound SARS-CoV-2 proteins with affinities similar to that of UFH, yet (Partridge, *et al.,* 2020), found that fondaparinux had no inhibitory effects on binding activity in RY4 cells. These *in vitro* studies reveal enoxaparin to be the most promising treatment of SARS-COV-2 in patients, with high inhibitory activity of viral cell binding and transmission. The heparin mimetic pixatimod also appears to be a potential therapeutic for SARS-CoV-2, however, more research is required for both agents in order to elucidate the interactions, dosage concentrations and inhibitory concentrations further.

Despite the research available, *in vitro* studies do not provide consistent evidence with regards to the inhibitory concentration of UFH and other heparin forms, furthermore, the degree of antiviral activity varies. As a result, additional study is required to further explicate the *in vitro* interactions of heparin, modified-heparins and mimetics and SARS-CoV-2, which may provide a basis for future therapeutic research and application.

Studies have shown that plant derived sulphated polysaccharides, can bind and inhibit SARS-CoV-2 *in vitro*. In contrast to studies with heparin, it appears that there is a relationship between level of sulphation and molecular weight and the inhibitory activity that the polysaccharides display (Jang, *et al*., 2021) (Song, *et al*., 2020). Furthermore, it has been demonstrated that *in vitro*, these polysaccharides could be more effective than current standard treatment, remsdesivir, as shown by (Jang, *et al*., 2021). λ-carrageenan was found to have a much lower EC50 than remsdesivir, 0.9 ± 1.1µg.ml-1 compared to an EC50 of 23.5 ± 1.2µM. Furthermore, λ-carrageenan had a much higher selectivity index of 333.3 compared to >12.8 in the case of remdesivir. This suggests that

λ-carrageenan is a promising potential antiviral therapeutic against SARS-CoV-2 requiring further research to investigate the effects of concentration and cytotoxicity as a basis for potential future clinical research. A similarly neglected area of research at this time, is the structure-function relationship between SARS-CoV-2 and other sulphated GAGs. Only one study was available at the time of writing that investigated this relationship, Mycroft-West, *et al*., 2020b, structural analysis by circular dichroism showed that all studied GAGs induced conformational change in the secondary structure of SARS-CoV-2. Further research is required to determine the exact nature of this conformational change and the effect it has on the infectivity of SARS-CoV-2. Despite promising research with regards to other enveloped viruses, currently there is little research available elucidating the activity of non-heparin derived sulphated carbohydrates from natural sources, such as plants. This is most likely a result of the urgency of the SARS-COV-2 pandemic and the need for already licensed treatments in order to allow for a faster approval and subsequent rollout. However, this limited research provides evidence to support the future research into these carbohydrates for potential use as an antiviral for SARS-CoV-2.

Clinical research has determined that that was already known that heparin can utilised as an effective treatment in patients with SARS-COV-2. When compared to other treatments, such as remdesivir and hydroxychloroquine, LMWH treatment was associated with reduced risk of death. With regards to composite end point LMWH had the lowest hazard ratio of all assessed treatments. Given that anticoagulation is the most researched target of SARS-COV-2 therapy with regards to heparin, it was imperative to determine that heparin is the most effective and safe form of treatment. Two studies included in this scoping review sought to compare the effectiveness of heparin treatment compared to other anticoagulation therapies such as apixaban. Both studies found that prophylactic enoxaparin is the most effective heparin form and dosage strategy, second to prophylactic apixaban. Billet, *et al.,* 2020, found that all forms of heparin treatment, prophylactic heparin and enoxaparin and increasing concentrations of the drugs were protective against mortality, except the highest concentration of heparin treatment, thrice daily, consistent with Rivera-Izquierdo, *et al*., 2020. These studies provide a clear basis for the use of heparin as a treatment in the case of SARS-COV-2, with mortality benefits only bested by apixaban, further research could focus on comparison between the two treatments to elucidate the mechanisms of action and their clinical consequences in both treatments in SARS-COV-2 patients. Being that heparin still provided reduced risk of mortality, research into effective treatment protocols, type of heparin and dosage are imperative to further elucidate the benefit of the treatment and to optimise the administration of it to have the most clinical impact.

Promising research has been carried out into the use of heparin as a therapeutic with anti-inflammatory properties against SARS-CoV-2, however, it is very limited with only 6 studies investigating this quality for treatment. The efficiency of heparin as an anti-inflammatory in the

case of SARS-CoV-2, an *in vitro* study was carried out to determine the interaction of low molecular weight heparin and IFNγ and IL-6. LMWH was found to bind IFNγ at the C-termini to form complexes that blocked the IFNγ transduction pathway. Furthermore, LMWH was found to inhibit the antiproliferative activity of IFNγ in a concentration dependent manner and abolish the activation of the JAK/STAT1α pathway. A clinical study showed that following heparin treatment, IL-6 levels of patients were significantly reduced, not only that but significantly lower than those who did not receive heparin treatment. Further studies showed that following LMWH treatment, the C reactive protein levels of patients were significantly decreased compared to untreated groups (D'Ardes, *et al*., 2020), (Gonzalez-Ochoa, *et al*., 2020) (Yormaz, *et al*., 2020). In contrast, a retrospective cohort study investigating inhibition of heparinase by dalteparin found no significant difference between IL-6 levels between the treatment and control group. Given the limited scope of the research into this application of heparin, the effects cannot be conclusively stated, however, it appears that heparin does have a beneficial anti-inflammatory role in clinical SARS-COV-2 patients, that acts to reduce the length of hospital stay and risk of complications.

Heparin is licensed for the use as an anticoagulant primarily for the prevention of venous thrombus formation, however, it has been identified as a treatment for heparin due to the coagulopathies that occur during viral infection. Heparin has been shown to decrease the incidence of mortality compared to control groups (no heparin treatment), with a greater protective effect in less severe SARS-COV-2 cases. Following this, research aimed to determine whether low molecular weight heparin could be used in a similar fashion and potentially greater benefit. Studies showed that heparin treatment is associated with greater risks of admission to the ICU, increased duration of hospital stay and a greater incidence of thrombotic complications when compared to low molecular weight heparins. LMWH treatment also appears to confer a greater survival benefit in more severely ill or elderly patients. This research shows that low molecular weight heparins may be a more attractive treatment for SARS-CoV-2 than the standard UFH treatments, suitable for moderate critically ill patients. Fondaparinux has had varied success *in vitro*, however, a retrospective cohort study revealed its potential as a treatment for SARS-COV-2 when comparing it to the more promising enoxaparin. It was found that patients in the fondaparinux cohort had a similar incidence of mortality to the enoxaparin group and that fondaparinux had a net clinical benefit over enoxaparin equal to 4.6, with reduced risk of ARDS development and VTE and bleeding events. The research shows that clinically, low molecular weight heparin is a more attractive candidate for SARS-COV-2 treatment, with greater benefits in terms of survival, hospital stay duration and major bleeding complications. Given the novelty of this virus, more research will need to be carried out to consolidate these early findings, to further investigate the most effective low molecular weight heparin treatment.

The most researched area of anti-coagulant treatment of SARS-COV-2 with heparin is treatment strategy: prophylactic versus therapeutic treatment and dosage concentrations to determine the most beneficial treatment protocol. Studies have found extremely varied results, in the case of unfractionated heparin, two studies showed no discernible mortality benefits from either prophylactic or therapeutic treatments (Bolzetta, *et al*., 2020) (Li, *et al*., 2020), however, two studies disagree, one of which, Motta, *et al*., 2020, found that heparin decreased the risk of mortality and the other, Trinh, *et al*., 2020 found the opposite, that heparin increased the risk. The effects of UFH on duration of hospital stay, mechanical ventilation and thrombotic complications are also inconsistent over the studies included in this review. Therefore, further trials and studies need to be carried out to determine the benefits of this mode of treatment. In the case of low molecular weight heparin, results are just as unclear, with two studies finding that therapeutic treatment reduces the risk of mortality, (Canoglu & Saylan, 2020) (Paolisso, *et al*., 2020), whereas Rentsch, *et al*., 2020, found that prophylaxis decreased the incidence of mortality and one study, Bertoldi Lemos, *et al*., 2020, found no statistical difference in mortality rates of the two treatment protocols. Two studies did not control for the type of heparin given, Ionescu, *et al*., 2020 and Ionescu, *et al*., 2020b, focussing solely on the net benefits of heparin anticoagulant treatment prophylactically or therapeutically in SARS-COV-2 patients compared to no treatment at all. In both cases it was found that rates of mortality were reduced, with therapeutic dosage being the most effective, with an 86% reduction of risk compared to 65% for the prophylactic group. Studies regarding dosage concentration are limited, with only three studies in this scoping review investigating the effects of dosage concentrations on mortality and other clinical parameters. Two studies, Jonmaker, *et al.,* 2020 and Martinelli, *et al.,* 2021, found that increasing dosage concentrations are associated with increased ICU free days and decreased ICU admission and mortality risks, however, Rivera-Izquierdo, *et al.,* 2020 found that increasing concentrations increased the mortality hazard ratios proportionally. At present, the most beneficial dosage strategy for heparin treatment is still yet to determined, however, prophylactic or therapeutic treatment confers no disadvantage when compared to no treatment at all. This provides a basis for further work in this area that should not be a detriment to the health of potential patients. Current advice states that in all hospitalised non-pregnant adults with SARS-CoV-2 a treatment dose of a low molecular weight heparin should be considered (National Institute for Health and Care Excellence, 2022). This treatment would act as prophylaxis for venous thromboembolism and should be administered within 14 hours of admission in adults with SARS-CoV-2 who need low-flow or high-flow oxygen, continuous positive airway pressure, non-invasive ventilation, or invasive mechanical ventilation, and who do not have an increased bleeding risk (National Institute for Health and Care Excellence, 2022). Current guidelines state that this treatment should be continued for a minimum of 7 days, even if the patient leaves the hospital setting to go home, this will continue in the form of self-administered subcutaneous injections (National Institute for Health and Care Excellence, 2022). Further research

could be directed towards the multi-therapeutic properties of heparin with regards to its anti-inflammatory and anti-viral properties that may identify heparin as a core treatment in a cocktail of drugs tailored to ameliorate the symptoms of SARS-CoV-2. In addition, the existing data for nebulised heparin in clinical trials focussing on asthma provide a strong basis for further research into the delivery of heparin, particularly with regards to self-administration as a number of patients may feel less comfortable using injections than they would using a nebuliser.

Overall, more research is required to understand the multi-faceted role of heparin treatment in causing differential outcomes and effects in clinical cases of SARS-COV-2. Due to the novelty of this virus and the urgency needed to find effective treatments, the focus of this review was largely based around the anticoagulant properties and benefits of heparin treatment in SARS-COV-2 patients. Although outside the scope of this review, the distinction of the most effective heparin type, concentration, and dosage strategy as well as the growth of the anti-inflammatory area of research and its effect on clinical SARS-COV-2 cases remain subject for elaboration and investigation in future studies.

There are several limitations to mention. Firstly, a large proportion, 19, of the included studies were 'grey literature', not peer-reviewed which increases the risk of bias in these studies. However, the impact of this should be limited since the outcome of this scoping review was based on the data provided, rather than the outcome as determined by the authors. Second, there may have been selection bias as this review may not have identified all the available data on the topic, meaning relevant studies were missed. Third, finding a balance between a search strategy that was neither to focussed nor too broad and adjusting the search throughout the process to accommodate new MeSH terms as they were found. Strengths of the study are the extensive systematic search aimed to include all studies which provided evidence for heparin as a potential treatment for SARS-COV-2. Second that numeric evidence was shown to strengthen the argument for heparin as a multi-modal treatment.

Concluding, in currently available studies on sulphated carbohydrates as a therapy for SARS-COV-2, only 4 turned the focus to non-heparin polysaccharides. This is a burgeoning area of research with great antiviral potential, yet the data is restricted by the current lack of interest in this topic. Similarly, only 6 studies focussed on anti-inflammatory properties of heparin, to provide a further understanding and greater basis for use as a therapy, further research should be pointed in this direction. It would be of great clinical relevance for further laboratory-based studies elucidating the structural and sulphation requirements for inhibition of SARS-COV-2 binding to occur to further optimise treatment, which could result in a more targeted treatment with less adverse coagulation effects. Furthermore, more trials should be undertaken to further evaluate the benefits of enoxaparin *in vivo*, as well as the mimetic fondaparinux as this review shows they show the greatest potential

as SARS-COV-2 treatments. Similarly, further study is required into the dosage strategy of treatment, with regards to prophylactic and therapeutic treatment to ensure that patients receive the greatest standard of care with the least risk.

Chapter 3: Materials and Methods

3.1. Rationale:

The literature demonstrates interactions of heparin with spike protein RBD, to confirm this interaction the effects of thermal stability was explored using differential scanning fluorimetry. DSF exploits conventional real time PCR machines to monitor the thermal denaturation of a protein through a range of temperatures, in the presence of a hydrophobic fluorescent dye. Upon heating, the hydrophobic regions of proteins become exposed upon unfolding, resulting in increased fluorescence. The midpoint of unfolding (T_m) can be determined through the first differential of the resulting melt curve and changes in T_m values in the presence of ligands can be used to determine binding affinity (Zhang & Monsma, 2010).

Research suggests the potential for sulphated plant compounds as antiviral agents against SARS-CoV-2. Plant polysaccharides, sea cucumber sulphated polysaccharide (SCSP), fucoidan and iota-carrageenan (ι-carrageenan) were assessed for their inhibitory activities in Vero cells (Song, *et al*., 2020). Of the four, sea cucumber sulphated polysaccharide showed the highest antiviral activity with IC_{50} of 9.10 µg.ml^{-1} (Song, *et al*., 2020). Further fucoidan and ι-carrageenan also showed inhibitory activity at concentrations of 15.6µg.ml^{-1} and ≥125 µg mL^{-1}, respectively (Song, *et al*., 2020). Further study by (Jang, *et al*., 2021), determined that λ-carrageenan inhibited viral infection of 293T cells at a lower concentration that remdesivir and with a much higher selectivity index. Research in this area is limited as a large proportion of SARS-CoV-2 research is directed towards repurposing pre-existing drugs. Considering the success of previous *in vitro* studies would be pertinent to further investigate the antiviral potential of sulphated plant compounds.

3.2. Materials

3,3'5,5'- tetramethylbenzidine (T5525-50TAB, Thermofisher)

Alfa Aesar ™ Streptavidin, streptomyces avidinii (Catalogue no.15464809; ThermoFisher)

Ampcillin (Catalogue no. A1000000, Sigma-Aldrich)

Biotinylated heparin (Catalogue no. 375054, Millipore)

Biotinylated-ACE2 (Catalogue no. 10108-H08H, Sino Biological)

Bovine serum albumin (fraction V). (Catalogue No. 05470, Sigma-Aldrich)

Brij35 (w/v) (Catalogue no. B4184, Sigma-Aldrich)

BugBuster™ (Catalogue no. 70584-M, Sigma-Aldrich)

Chloramphenicol (Catalogue no. C3175, Sigma-Aldrich)

Dimethylsulfoxide, anhydrous, ≥99.9%. (Catalogue No. 276855, Sigma-Aldrich)

DNAase (Catalogue no. EN0521, Thermofisher)

Donkey anti-Rabbit IgG (Catalogue no. 406421, BioLegend)

Fibrinogen (Catalogue no. F3879, Sigma-Aldrich)

H_2O_2 = (Catalogue no. H1009, Sigma-Aldrich)

H_2SO_4 (Catalogue no. S25898, ThermoFisher)

HPLC (analytical) grade H2O. (Catalogue No. 11307090, Fisher)

Hydrogen peroxide (Catalogue no. H1009, Sigma-Aldrich)

IMAC Sepharose® High Performance (Catalogue no. GE16-0920-07, Sigma-Aldrich)

Imidiazole (Catalogue no. 15513, ThermoFisher)

LB Broth (Catalogue no. 10855001, ThermoFisher)

Lysozyme (Catalogue no. 90082, ThermoFisher)

MagicMedia™ (Catalogue no. K6803, Thermofisher)

Phosphate buffered saline = (Catalogue no. NAT1006, SLS)

Phosphate citrate buffer (Catalogue no. P4809, Sigma-Aldrich)

Porcine mucosal heparin sodium salt (1000 I.U.ml-1 (Catalogue no. FP1086, Wockhardt)

Porcine mucosal heparin sodium salt (201 IU.mg-1). (Catalogue No. PH03004, Celsus)

ProteOrange (Catalogue no. 41210, Universal Biologicals)

pRSET A, B & C Bacterial Expression Vectors (Catalogue no. V35120, Thermofisher)

Rabbit-SARS-CoV-2 (2019-nCoV) Spike RBD Antibody (Catalogue no. GTX01546-PRO-GTX, Stratech)

SARS-CoV-2 RBD, UK mutation B.1.1.7 (Catalogue no. p1-spike-v2, InvivoGen)

SARS-CoV-2 Spike Protein, residues 330−583 (GenBank: MN908947) (Catalogue no. 100987)

SHuffle® T7 Express Competent E. coli (Catalogue no. C3029, NEB).

Sodium bicarbonate. (Catalogue No. 10244683, Fisher)

Sodium chloride 99.5%. (Catalogue no. 10092740, Fisher).

Sodium phosphate (Catalogue no. S0751, Sigma-Aldrich)

SYPRO® Orange Protein Gel Stain (Catalogue No. S5692, Sigma-Aldrich)

Urea (Catalogue no. 603430, Sigma-Aldrich)

3.3 Equipment

Greiner high binding 96 well plates ELISA (Catalogue no. 655061, Greiner)

MicroAmp® optical 96-well reaction plate. Fisher, Sweden, Catalogue No. 10411785

MicroAmp™ optical adhesive film. Fisher, Sweden. Catalogue No. 10567414

Sephadex G-25 column (G2580, Sigma-Aldrich)

Step One Plus RT-PCR machine. Fisher, Sweden.

Tecan Infinite M200 Multiwell plate reader. Tecan, Switzerland. Catalogue No. M200

3.4. Methods

Protein expression:

Starter Culture:

A starter culture was produced directly from glycerol stocks of residues 330−583 of the SARS-CoV-2 Spike Protein (GenBank: MN908947) cloned upstream of a N-terminal 6XHisTag in the pRSETA expression vector and transformed into SHuffle® T7 Express Competent E. coli (NEB, UK). Recombinant SARS-CoV-2 Spike protein was incubated with 20mL of autoclaved LB broth containing 20μL of both ampicillin and chloramphenicol and Incubated for 5 hours at 37°C. Auto-inducible expansion: MagicMedia™ (Thermofisher) was used according to protocol: components A:B used in a 20:1 ratio to form a 200mL solution. Antibiotics: 20μL of both ampicillin and chloramphenicol were added. Media was incubated with starter culture and antibiotic at 30°C and shaking at 250rpm for ~16 hours.

Cell Collection

Media centrifuged for 10 minutes at 10,000 x g. Cells resuspended in ice cold PBS (1x) and recentrifuged. Cell pellets frozen for later use.

Cell lysis:

Pellets were thawed then resuspended in lysis buffer, BugBuster™ (5 grams per gram of cell paste), DNAase (125 units per gram of cell paste) and lysozyme (5KU per gram of cell paste) at room temperature. Resuspended cells were incubated on a rocker at room temperature for 30 minutes until solubilised. Following this the suspension was centrifuged at 16,000 x g for 20 minutes at 4°C

to remove insoluble material. Supernatant and cell pellet saved for further processing and for analysis by SDS-PAGE.

Protein Purification:

Inclusion body wash:

Pellet was resuspended in the same volume of BugBuster™ as used previously, suspension was pipetted to ensure a high purity preparation. Lysozyme was added to the suspension to a final concentration of 1KU/mL and gently vortexed, then incubated on a rocker at room temperature for 5 minutes. A solution of diluted BugBuster™ and deionised water in a 1:10 v/v ratio was prepared and added in equal volumes to the inclusion body solution and vortexed. The solution was then centrifuged for 15 minutes at 15,000 x g at 4°C, supernatant was removed using a pipette to collect the inclusion bodies. Inclusion bodies were resuspended in diluted BugBuster™, 10mL per gram of cell paste, supernatant was removed using a pipette. Solution was resuspended a total of three times. On the final wash, a small sample of the suspension was removed for SDS-PAGE analysis. The remaining inclusion body solution was centrifuged at 16,000 x g for 15 minutes at 4°C, and the supernatant was removed.

Protein denaturation:

Inclusion body was resolubilised in 5mL denaturing buffer (50 mM NaH_2PO_4, 300 mM NaCl 5 mM imidiazole, 8 M urea, 1 mM β-mercaptoethanol, pH 7.4) and incubated at room temperature on a rocker until dissolved, ~1 hour. Suspension was centrifuged at 10,000g for 10 minutes to remove insoluble components, a small sample of the suspension was saved for SDS-PAGE analysis.

Protein Refolding:

On Column Refolding:

Protein in denaturing buffer was applied to IMAC Sepharose media (Co+) and incubated on a rocker at room temperature for 1 hour. Denaturing buffer in a stepwise gradient of urea (6-0M urea), 3 column volumes for each concentration. Flow through was collected and stored. Following extensive washing, protein was eluted using 20mM NaH_2PO_4 pH 8, 300 mM NaCl and 500mM imidiazole. Fractions were pooled and buffer-exchanged to phosphate-buffered saline (PBS; 140 mM NaCl, 5 mM NaH2PO4, 5 mM Na2HPO4, pH 7.4; Lonza, UK) using a Sephadex G-25 column (GE Healthcare, UK). Recombinant protein was stored at -20°C until required.

Enzyme linked immunosorbent assay:

ELISA assay for measuring inhibition of RBDACE2 binding by heparin and plant compounds

To explore the antiviral activity of plant polysaccharides, a library of 29 variably sulphated plant carbohydrates were screened using an enzyme-linked immunosorbent assay to determine their inhibitory activity. The inhibitory activity of heparin was also assayed in this manner to act as a comparison, similarly, wells lacking ACE2 were used to represent 100% inhibition, whereas full length spike protein with ACE2 was used to represent 0% inhibition. Streptavidin, reconstituted at 1mg/mL in 1x PBS (3µgml^{-1}) in 50mM sodium carbonate buffer pH 9.5 (50µl per well) was incubated for 1 hour at 37°C in high binding 96-well plates. Plates were washed three times with 1 xPBS 0.2% Brij35 (w/v) and then blocked for 1 hour at 37°C with PBS 0.2% Brij35 (w/v) + 1% casein (w/v). Plates were washed three times again using 1xPBS 0.2% Brij35 (w/v) and then incubated with biotinylated ACE2 in PBS 0.2% Brij35 (w/v) + 1% casein (w/v) for 1 hour at 37°C. plates were then washed three times with PBS 0.2% Brij35 (w/v). RBD was incubated separately in PBS 0.2% Brij35 (w/v) + 1% casein (w/v) for 30 minutes at room temperature with or without heparin before addition to prewashed plates containing immobilised ACE2. Plates were incubated for 1 hour at 37°C, before being washed three times with 1xPBS 0.2% Brij35 (w/v). Bound spike protein was detected following incubation with 0.5µgml^{-1} Rabbit-SARS-CoV-2 Spike RBD antibody (Stratech) in 1xPBS 0.2% Brij35 (w/v) + 1% casein (w/v) for 1 hour at 37°C. Plates were washed three times with 1xPBS 0.2% Brij35 (w/v) before a 30-minute incubation at 37°C with HRP-conjugated Donkey anti-Rabbit IgG diluted to 1:1000 in 1xPBS 0.2% Brij35 (w/v) + 1% casein (w/v). Plates were washed five times using PBS 0.2% Brij35 before being developed with 3,3'5,5'- tetramethylbenzidine, prepared in 1mL of DMSO, 9 mL of 0.05 M phosphate citrate buffer, pH 5.0 and 2 µL H$_2$O$_2$. Plates were incubated until development of colour, at which point the reaction was stopped by the addition of 20µL 2M H$_2$SO$_4$. Well absorbances were determined at λ=450nm using a Tecan infinite M200 Pro plate reader.

Differential Scanning Fluorometry:

Identification of optimal dye:

To determine the optimal fluorescent dye to use for RBD binding investigations, a preliminary assay using fibrinogen and lysozyme was carried out. The hydrophobic dye SYPRO Orange (1.25x, Invitrogen) was compared to the generic version of the dye, ProteOrange.

Determination of heparin binding:

Differential scanning fluorimetry (DSF) was performed using the hydrophobic dye SYPRO Orange (1.25x; Invitrogen) at a concentration of 25x to examine the thermal denaturation of RBD UK variant, 1µg per well in the presence of 200µg heparin (Celsus or Wockhardt) and alone, in PBS pH 7.6, with a total well volume 40 µl. Control wells containing H$_2$O or heparin (1µg per well)

(Celsus or Wockhardt) without the RBD Spike protein were also screened to ensure that melting temperature changes were not a result of interactions between SYPRO Orange and the carbohydrate ligand. Melt curve experiments were performed in 96-well qPCR plates (AB Biosystems) using an AB biosystems StepOne plus qPCR machine with the TAMRA filter setting enabled. Samples were incubated at 25°C for 2 minutes, increasing sequentially by 0.5°C increments every 30 seconds up to 90°C. Melt curves were smoothed (nine neighbours, second-order polynomial, Savitzky-Golay) and first differential plots were constructed using Prism 8 (GraphPad). The peak of the first-differential plots was used to calculate Tm values.

Chapter 4: Results

4.1. Experimental design and optimisation:

Prior to investigation of heparin and sulphated plant compounds with the receptor binding domain of SARS-CoV-2, extensive experimental optimisation was carried out. These investigations were done using both fibrinogen and lysozyme, both are accessible and reliable proteins for use in differential scanning fluorimetry. This was to identify the optimal concentration of ligand and protein to ensure the clarity and accuracy of the results whilst ensuring that the limited resource of RBD was not unnecessarily used in high concentrations. Further, two fluorescent dyes were analysed to determine the optimal dye for use in the final investigations, the standard SYPRO Orange was compared with a newer, more affordable dye, ProteOrange. SARS-COV-2 research is expansive, time consuming and at times costly, if ProteOrange could be used for thermal stability analysis it could decrease costs allowing a larger budget for other reagents.

4.1.1. Differential Scanning Fluorimetry analysis of lysozyme and fluorescent dyes

The differential scanning fluorimetry spectra of lysozyme at varying concentrations, 400µg/mL, 200µg/mL and 100µg/mL, and SYPRO Orange or ProteOrange at concentrations of 50x, 25x, 12.5x, 6.25x, 1.5625x. A final spectrum representing no lysozyme, no SYPRO Orange and no lysozyme and SYPRO Orange was included to act as a control. These results are shown in *Figures 11* and *12* respectively. The physiological action of lysozyme occurs in the granules of neutrophils macrophages and in serum, with optimal activity in the pH range of 6.5-7.5, to represent this environment PBS was used.
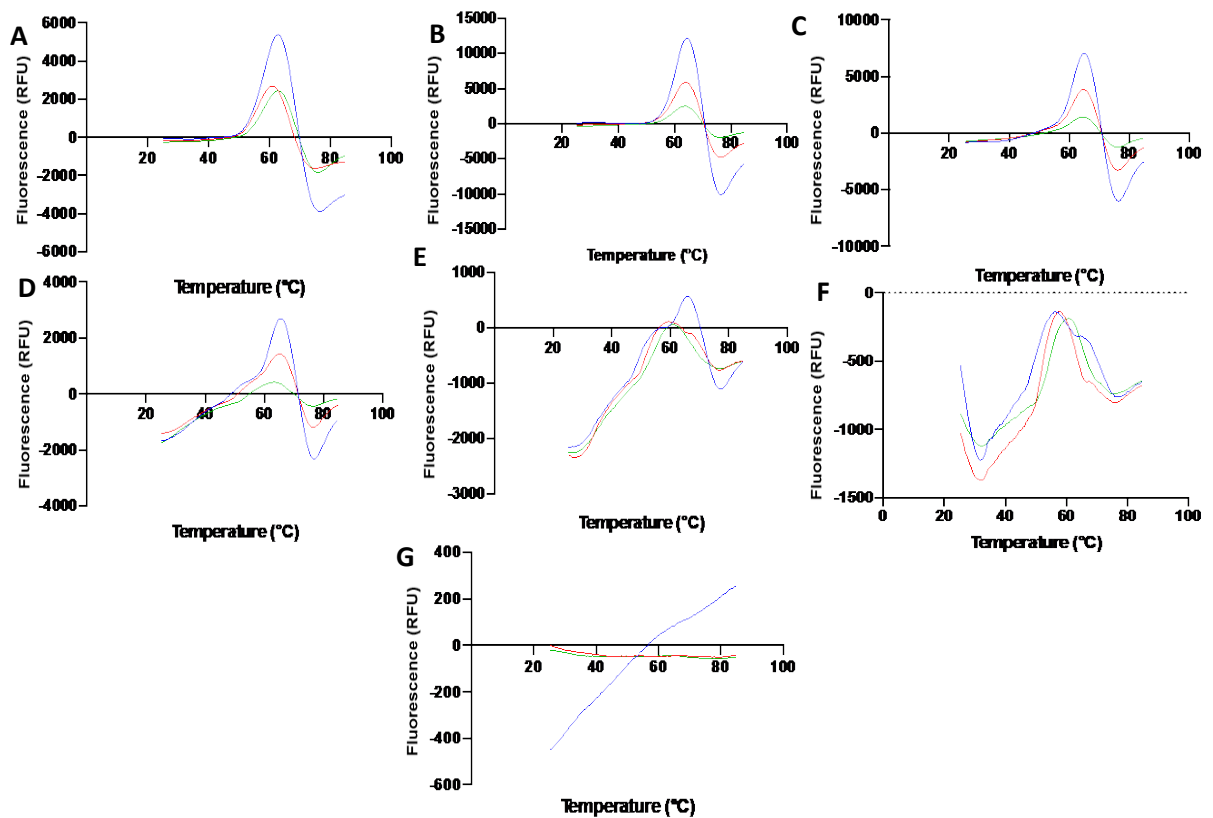
Figure 11: Determination of optimal SYPRO Orange and lysozyme concentration: *the thermal stability of lysozyme at varying concentrations 400μg/ml (blue), 200μg/ml (red) and 100μg/ml (green) was measured using decreasing concentrations of SYPRO Orange: 50x (A), 25x (B), 12.5x (C), 6.25x (D), 3.125x (E) and 1.5625x (F). Controls (G): blue: no lysozyme, red: no SYPRO and green: no SYPRO or protein. Optimal concentration of lysozyme is 400μg/ml with the optimal concentration of SYPRO Orange being 25x as seen in B.*

Figure 11A shows the spectra of lysozyme with SYPRO Orange at 50x, at this concentration of SYPRO Orange the highest fluorescence value is produced when lysozyme is at a concentration of 400μg/mL. At this concentration, a peak was produced at ~6000RFU, compared to ~2800RFU and ~2400RFU at concentrations of 200μg/mL and 100μg/mL respectively. The concentration of lysozyme at which the largest peak, therefore largest emission of fluorescence was produced, is 400μg/mL regardless of SYPRO Orange concentration. Figure 11B contains the optimal protein SYPRO Orange concentration, 25 x, with fluorescence values almost double that seen in 11A. At 400μg/mL fluorescence was ~12500RFU, compared to ~6000RFU at 200μg/mL and ~2500 at 100μg/mL. A decrease in concentration by half at 25 x, resulted in an almost 50% decrease in fluorescence at all concentrations, with fluorescence values of ~7000RFU at 400μg/mL, ~3700RFU at 200μg/mL and ~1300RFU at a concentration of 100μg/mL. This trend continues, as SYPRO Orange concentration decreases to 12.5x, the fluorescence emitted decreases, as seen in Figure 11D with values of ~2700RFU at 400μg/mL, ~1700RFU at 200μg/mL and ~400RFU at 100μg/mL. Figure 11E, shows the concentration of SYPRO Orange at which the thermal stability could not be accurately measured, with fluorescence values of approximately 600RFU at 400μg/mL, ~125RFU at 200μg/mL and ~30RFU at 100μg/mL. SYPRO Orange concentration of 6.125x.
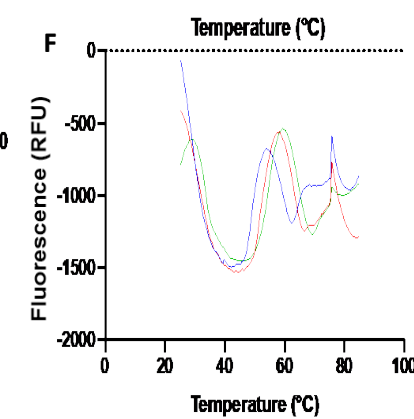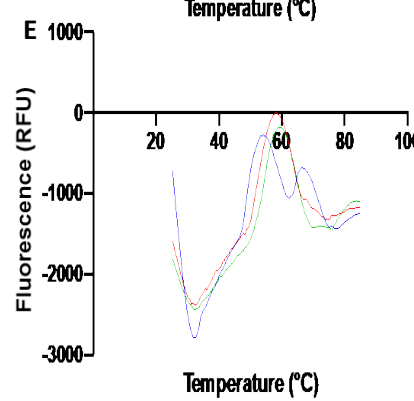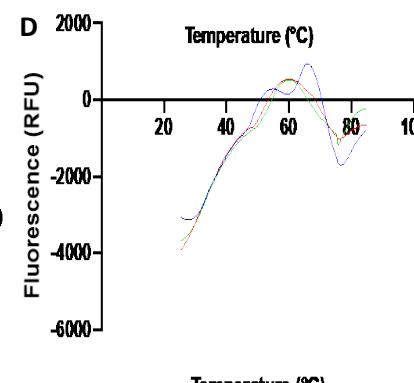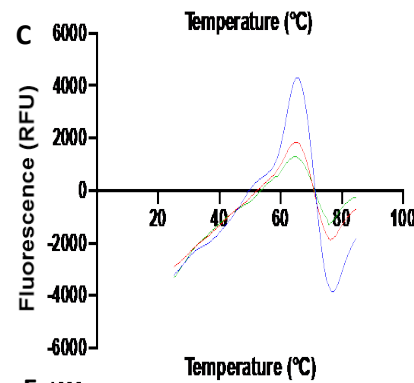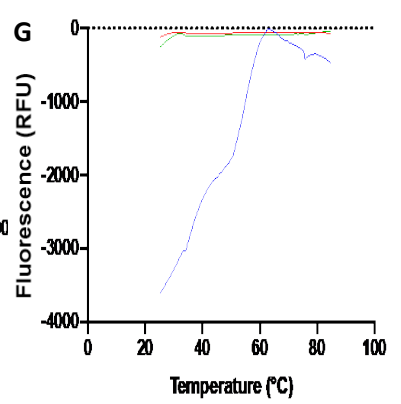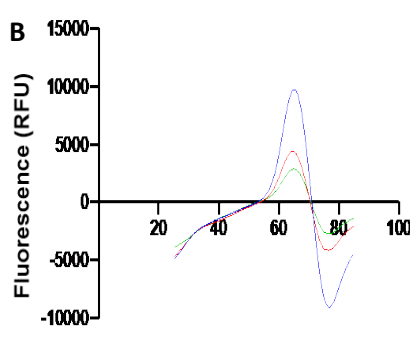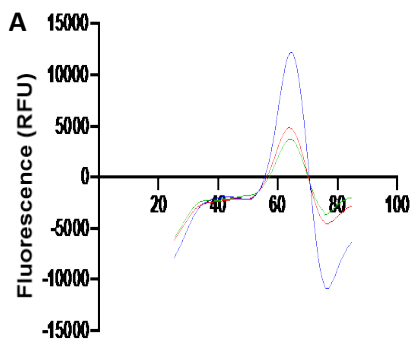
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Figure 12A shows the spectra of lysozyme with ProteOrange at 50x, at this concentration of ProteOrange the highest fluorescence value is produced when lysozyme is at a concentration of 400μg/mL. At this concentration, a peak of ~125000RFU was produced, compared to ~5000RFU and ~3500RFU at concentrations of 200μg/mL and 100μg/mL respectively. The optimal concentration of lysozyme is 400μg/mL, at this concentration the emission of fluorescence is at its highest, with the exception of Figures E and F- concentrations of ProteOrange of 3.125x and 1.5625x. However, these results are negligible as the spectrum produced at these concentrations are not suitable for use for analysis of protein stability. As the concentration of ProteOrange decreases, as does the fluorescence emission values upon protein unfolding. As shown in Figure 11B, at a concentration of 25x, the fluorescence decreased, with values of ~10000RFU, ~4000RFU and ~2500RFU at concentrations of 400μg/mL, 200μg/mL and 100μg/mL respectively. As the concentration of ProteOrange halves, the fluorescence decreased proportionally, Figure 11C, with fluorescence values at 400μg/mL of ~4700RFU, ~1800RFU at 200μg/mL and ~1000RFU at 100μg/mL. At a ProteOrange concentration of 12.5x the spectrum becomes unusable for analysis, with fluorescence values of ~900RFU at 400μg/mL, ~500RFU at 200μg/mL and 100μg/mL. Figures 11E and F, show the concentration of ProteOrange at which the thermal stability could not be accurately measured, as the spectra showed negative fluorescent values.

4.1.2. Differential Scanning Fluorimetry analysis of fibrinogen and fluorescent dyes

The differential scanning fluorimetry spectra of fibrinogen at varying concentrations, 400μg/mL, 200μg/mL and 100μg/mL, and SYPRO Orange or ProteOrange at concentrations of 50x, 25x, 12.5x, 6.25x, 1.5625x. A final spectrum representing no fibrinogen, no SYPRO Orange and no lysozyme and SYPRO Orange was included to act as a control. These results are shown in Figures 13 and 14 respectively.
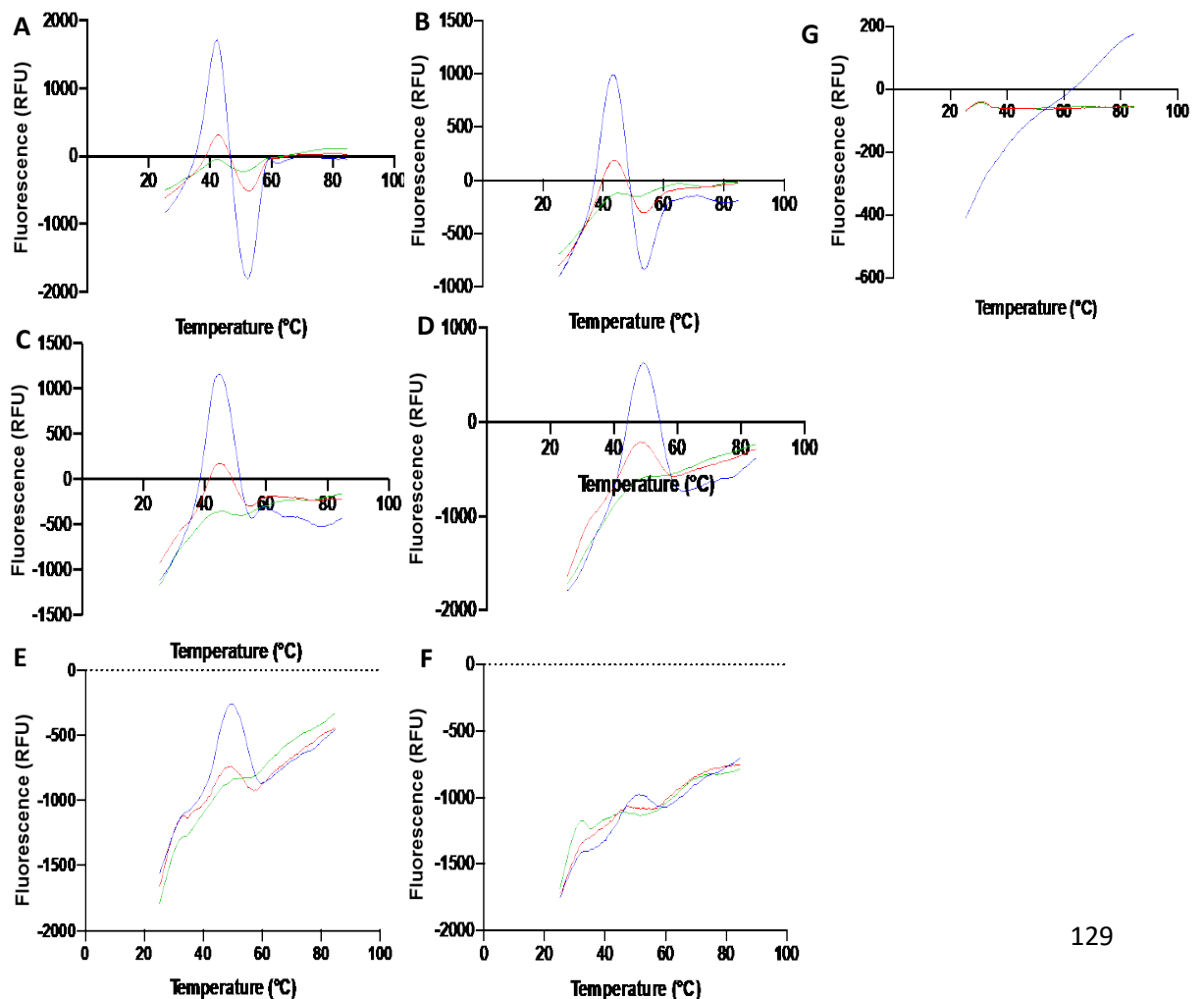

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Figure 13: Determination of optimal SYPRO Orange and fibrinogen concentration: *the thermal*

Figure 13 shows the spectra of fibrinogen with SYPRO Orange at decreasing concentrations, across the data it is evident that the optimal concentration of fibrinogen is 400μg/mL. Figure 13A shows the concentration of SYPRO Orange that is the most effective for protein thermal stability analysis, 50x. At this concentration, a peak was produced at ~1600RFU compared to ~300RFU at 200μg/mL at this concentration of SYPRO Orange there was no fluorescence emitted at 100μg/mL of fibrinogen. Figure 11B shows the spectra of the thermal stability of fibrinogen at 25x SYPRO Orange, with the fluorescence decreasing with the concentration of fibrinogen, ~1000RFU, ~200RFU and negative fluorescence at 400μg/mL, 200μg/mL and 100μg/mL. Interestingly, the fluorescence at 12.5x SYPRO Orange and 400μg/mL fibrinogen, as seen in Figure 13C was increased compared to 25x. With a fluorescence of 1250RFU, compared to 200RFU at 200μg/mL, with negative fluorescence again at 100μg/mL. When the concentration halved to 6.25x, the fluorescence emission sharply decreased at all concentrations of fibrinogen, 600RFU at 400μg/mL, and negative fluorescence emission at both 200μg/mL and 100μg/mL. The decrease in SYPRO Orange concentrations to both 3.125x and 1.5625x resulted in negative fluorescence values for all concentrations of fibrinogen.
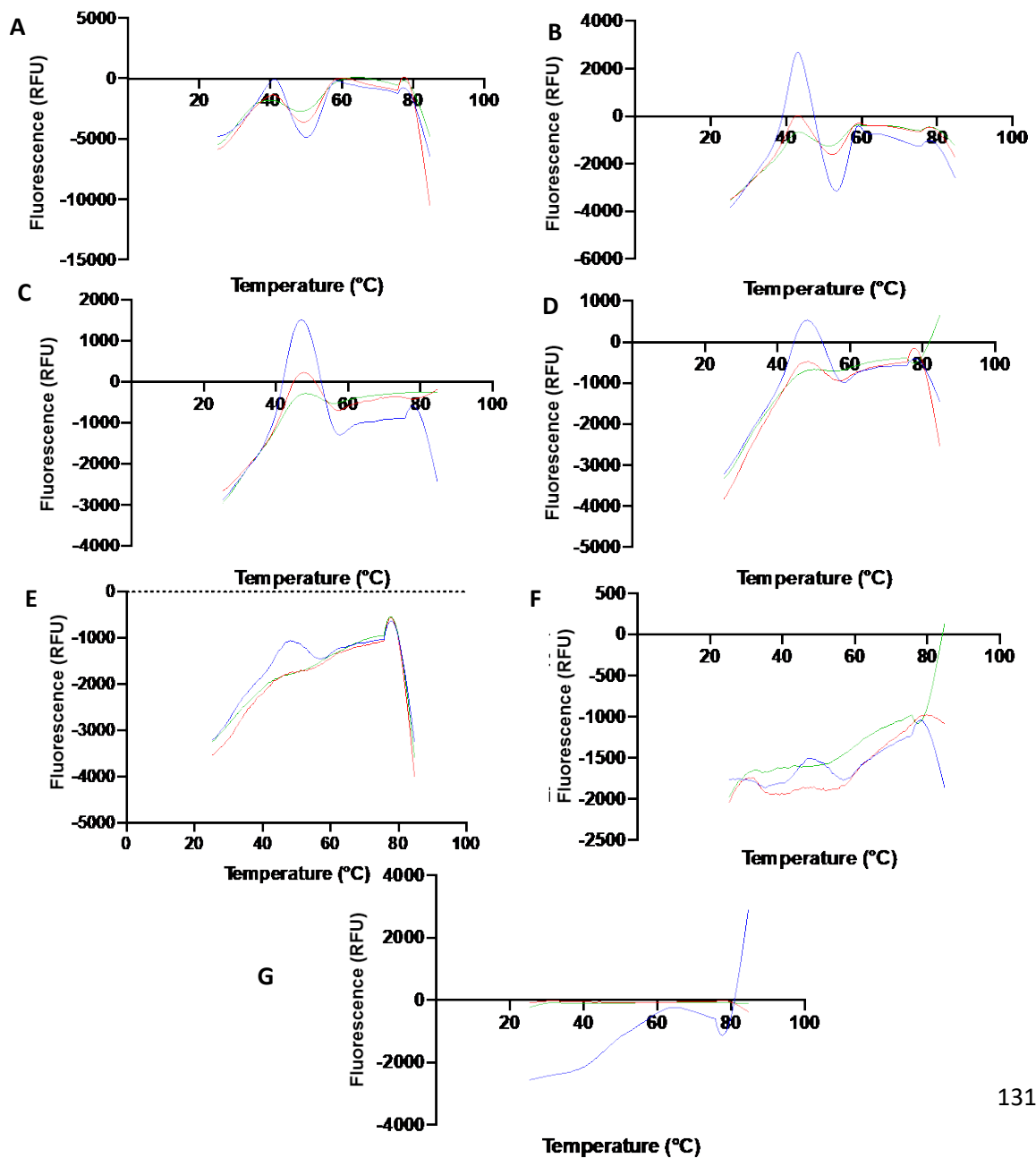
131

Figure 14: Determination of optimal ProteOrange and fibrinogen concentration: the thermal stability of

Figure 14 shows the spectra of fibrinogen with ProteOrange, as displayed the utility of ProteOrange with this protein is limited compared to lysoszyme, Figure 12. At a concentration of 50x ProteOrange, the fluorescence emission was negative in all cases. When the concentration halved, 25x, only 400 μg/mL produced a positive melt curve. Figure 14B shows optimal concentration of fibrinogen, 400 μg/mL and ProteOrange, 25x, the with a melting point emission of ~2800RFU. Similarly, when halved again, 12.5x ProteOrange, the optimal fibrinogen concentration was 400μg/mL, with an emission of ~1400RFU, compared to ~200RFU at a fibrinogen concentration of 200μg/mL. At a ProteOrange concentration of 6.25x, the fluorescence emission at 400μg/mL of fibrinogen was greatly diminished, at ~400RFU, the emission of lower fibrinogen concentrations was negative. At concentrations below 6.25x, the fluorescence emitted was too low for experimental use.

Figure 15: Determination of optimal fluorescent dye and concentration with lysozyme at optimal concentration: the thermal stability of lysozyme at a concentration of 400μg/ml was measured using varying concentrations of dyes SYPRO Orange (blue) and ProteOrange (red). A) 50x SYPRO and ProteOrange, B) 25x SYPRO and ProteOrange, C) 12.5X SYPRO and ProteOrange, D) 6.25x SYPRO and ProteOrange E) 3.125x SYPRO and ProteOrange F) 1.5625x SYPRO and ProteOrange. The best signal at the lowest possible

Figure 15 demonstrates the comparison of the optimal fluorescent dye with lysozyme at optimal concentration, 400µg/mL. Figure 15A shows the fluorescence emitted at the melting point of lysozyme when dyes are at a concentration of 50x, at this concentration, ProteOrange produced the highest emission, ~12500RFU compared to SYPRO Orange, ~6000RFU. As the concentration of dyes halved, 15B, the difference between fluorescence emitted was less notable, SYPRO Orange, ~12500RFU, compared to ~8000RFU with ProteOrange. As the concentration of fluorescent dye decreased, the emission produced decreased, with ProteOrange always having a lower fluorescence emission value. At a concentration of 12.5x, the emission produced by SYPRO Orange almost halves, ~7500RFU, similarly, a great reduction is seen in the fluorescence produced by ProteOrange, ~4000RFU. Figure 15D shows the dyes at a concentration of 6.25x, at this concentration the reduction in emission is noteworthy, with an emission of ~2900RFU with SYPRO Orange. Interestingly, at this concentration ProteOrange produced two melting points, the first at ~200RFU and the second at ~750RFU. At a concentration of 3.125x only SYPRO Orange produced a positive melting point, ~500RFU.
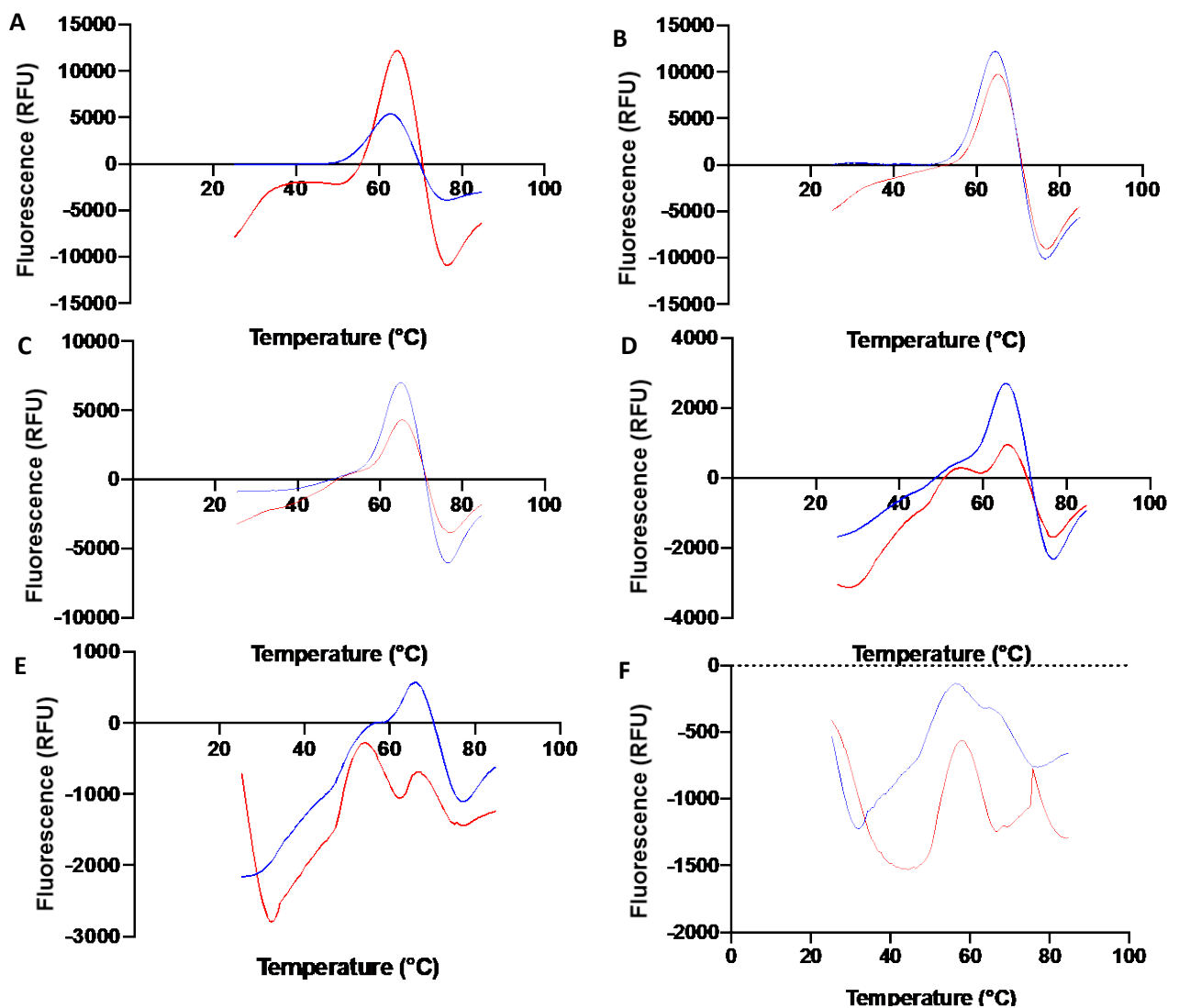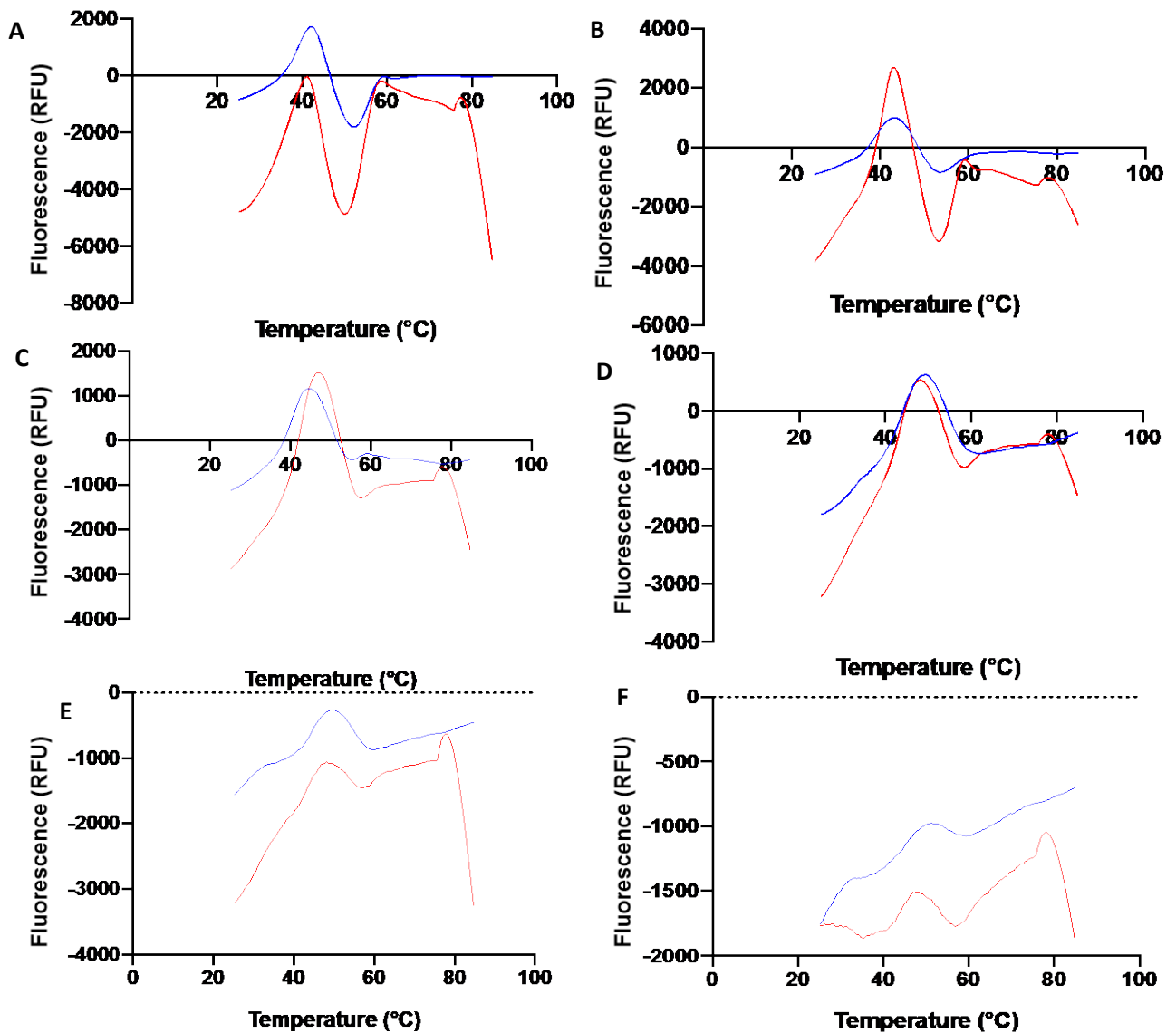
Figure 16: Determination of optimal fluorescent dye and concentration with fibrinogen at optimal concentration: *the thermal stability of fibrinogen at a concentration of 400µg/ml was measured using varying concentrations of dyes SYPRO Orange (blue) and ProteOrange (red). A) 50x SYPRO and ProteOrange, B) 25x SYPRO and ProteOrange, C) 12.5X SYPRO and ProteOrange, D) 6.25x SYPRO and ProteOrange E) 3.125x SYPRO and ProteOrange E) 1.5625x SYPRO and ProteOrange. The best signal at the lowest possible concentration, the optimal concentration, was at a concentration of 25x. At this concentration, the optimal signal was produced by ProteoOrange (red).*

Dissimilar to Figure 15, when using fibrinogen, a larger emission of fluorescence was produced by SYPRO Orange at a concentration of 50x, ~1700RFU, than by ProteOrange, which had a negative fluorescence emission. Despite this, as seen in 16B, ProteOrange at a concentration of 25x had a larger emission of fluorescence, ~2700RFU compared to SYPRO Orange, ~1000RFU. As the concentration decreased, the distinction between the two dyes decreased, at 12.5x there was approximately 300RFU difference in fluorescence emission, with ~1300RFU and ~1600RFU for SYPRO Orange and ProteOrange respectively. At 6.25x, the difference between the two is minimal, with an emission of ~500RFU produced by SYPRO Orange and ~400RFU by ProteOrange. With the concentration halved once more, 3.125x, the fluorescence emitted was unsuitable for experimental use, as the values were negative. With regards to fibrinogen, the optimal fluorescent dye is ProteOrange at a concentration of 25x, compared to lysozyme, where the optimal dye is 25x SYPRO Orange.
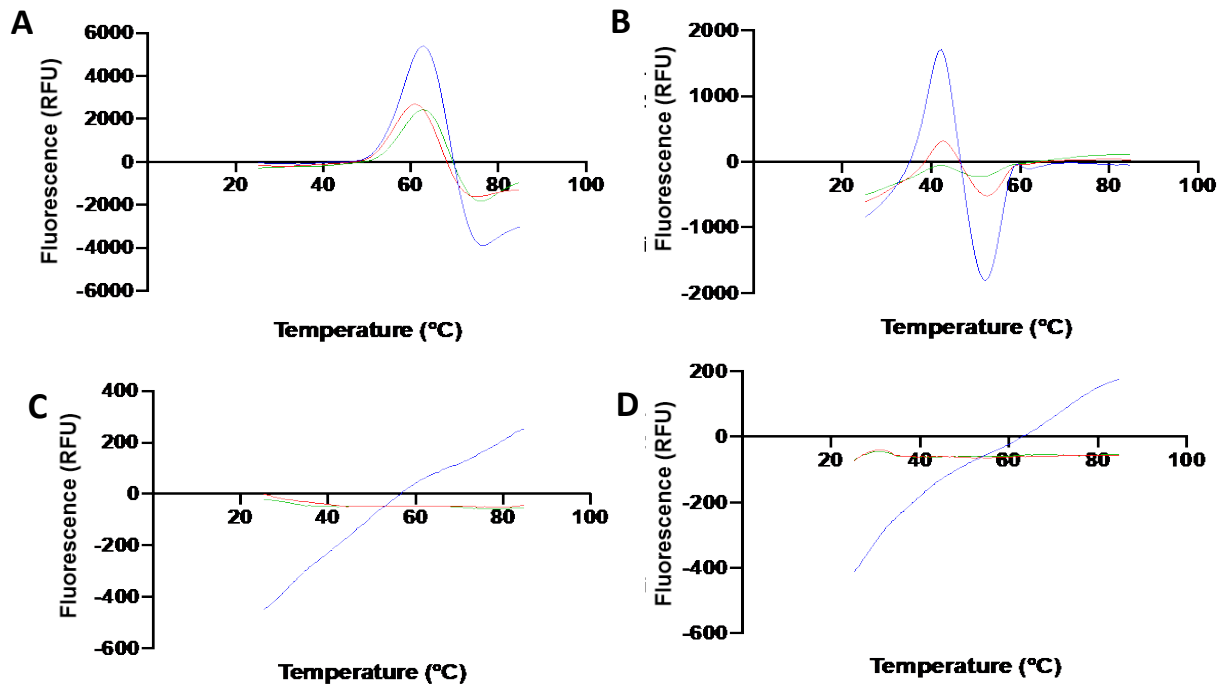
Figure 17: Comparison of fibrinogen and lysozyme with SYPRO Orange: *A) Blue = 400 µg/ml lysozyme 50x SYPRO, Red = 200 µg/ml lysozyme 50x SYPRO, Green = 100 µg/ml lysozyme 50x SYRPO. B) Blue = 400 µg/ml fibrinogen 50x SYPRO, Red = 200 µg/ml fibrinogen 50 x SYPRO, Green = 100 µg/ml fibrinogen 50x SYPRO. C) Blue = No lysozyme control, Red = No SYPRO control, Green = No lysozyme or SYPRO control. D) Blue – No fibrinogen control, Red = No SYPRO control, Green = No fibrinogen or SYPRO control. Both lysozyme and fibrinogen show the strongest fluorescence at 400µg/mL when fluorescent dye is at a concentration of 50x, as seen in figure A.*

SYPRO Orange was determined to be the optimal dye for use in differential scanning fluorimetry, figure 17 shows that the most effective protein with this dye is lysozyme. As seen in both figure A, at a concentration of 400μg/mL and 50x SYPRO Orange, an emission of ~5100RFU was produced, when this concentration is halved, 200 μg/mL, the fluorescence emitted decreases by almost 50%, ~2900RFU. At a concentration of 100 μg/mL, the emission of fluorescence does not decrease drastically, ~2500RFU. Comparatively, at a concentration of 400 μg/mL, fibrinogen emits a fluorescence of only, ~1750RFU, when this concentration is halved, 200 μg/mL, the emission of fluorescence is ~250RFU. At a concentration of 100 μg/mL, the fluorescence emission is negative, indicating this is an unsuitable concentration for experimental use.

4.1.3. Differential scanning fluorimetry: Heparin binds SARS-CoV-2 Receptor Binding domain
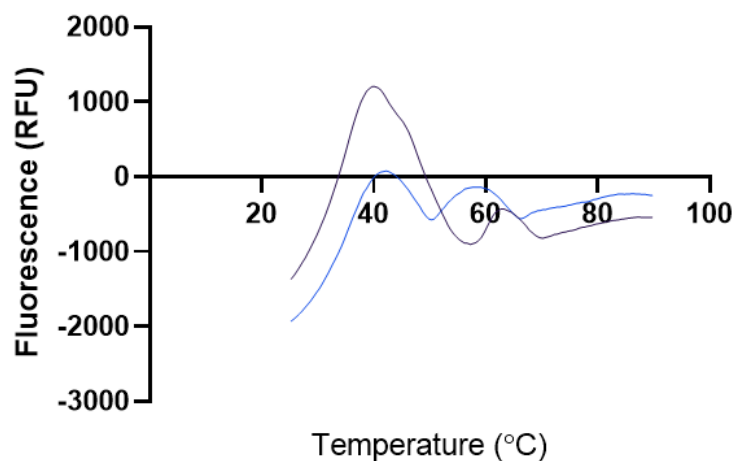
Figure 18:   Unfractionated heparins (UFs) interact directly with UK SARS-CoV-2 RBD domain. Differential scanning fluorimetry was employed to measure the thermal stability curve for recombinant SARS-CoV-2 RBD (UK mutation B.1.1.7) in the absence or presence of UF heparin. First differential of the thermal stability was plotted of 1μg SARS-CoV-2 RBD alone (blue) or with 200μg UF heparin (black). RBD alone has a T_m of 42.6°C and SARS-CoV-2 RBD in the presence of heparin has a T_m of 40.01°C.

The result of the interaction between heparin and SARS-CoV-S1 is documented in Figure 18, with a decrease in thermal stability of the RBD in the presence of heparin, 42.6°C and RBD and heparin 40.01°C, a decrease of 2.5°C. This indicates that the secondary structure of SARS-CoV-S1 is altered in response to heparin binding, which may affect the interaction of the RBD *in vivo*. Further, the difference between fluorescence values may be of note, fluorescence increases proportionally to unfolded protein abundance. This indicates that the level of unfolding increases in the presence of heparin, which affirms the destabilising nature of the interaction between SARS-CoV-S1 and heparin.
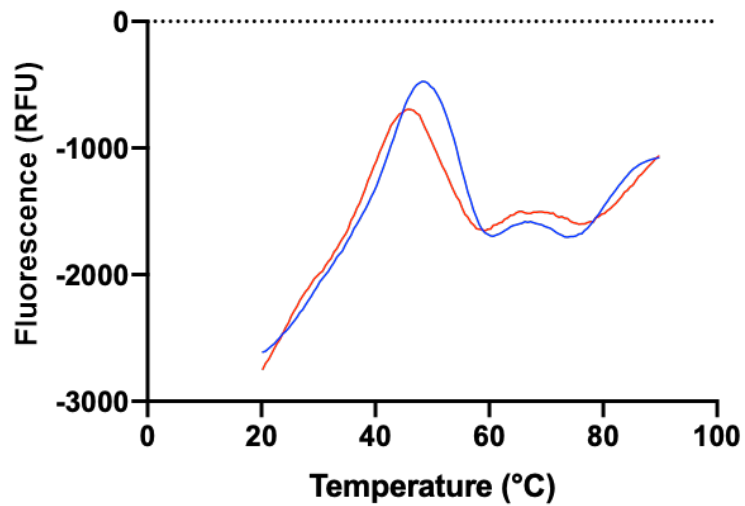
Figure 19: Unfractionated heparins (UFs) interact directly with SARS-CoV-2 spike S1 RBD domain (GenBank: MN908947***). Differential scanning fluorimetry was employed to measure the thermal stability curve for recombinant RBD in the absence (blue) or presence of UF heparin (1mg/mL)***

Similarly, using the SARS-CoV-2 spike S1 RBD, the thermal stability of the receptor binding domain decreases in the presence of heparin, 48°C compared to 50°C without any ligand binding, as seen in Figure 19. This indicates a change in the secondary structure that may affect the binding capabilities *in vivo*. However, the emission of fluorescence is slightly lower, 750RFU compared to 450RFU in the presence of heparin, this indicates that there was a larger unfolding event occurring when the RBD is alone in solution.

4.1.3. ELISA: Heparin and sulphated plan compounds inhibit SARS-CoV-2 binding to ACE2

Figure 20: Competitive ELISA assay to measure inhibition of binding of SARS-CoV-2 Spike S1-RBD in the presence of 24 various plant compounds. Using biotinylated human ACE2 protein immobilised on streptavidin coated pla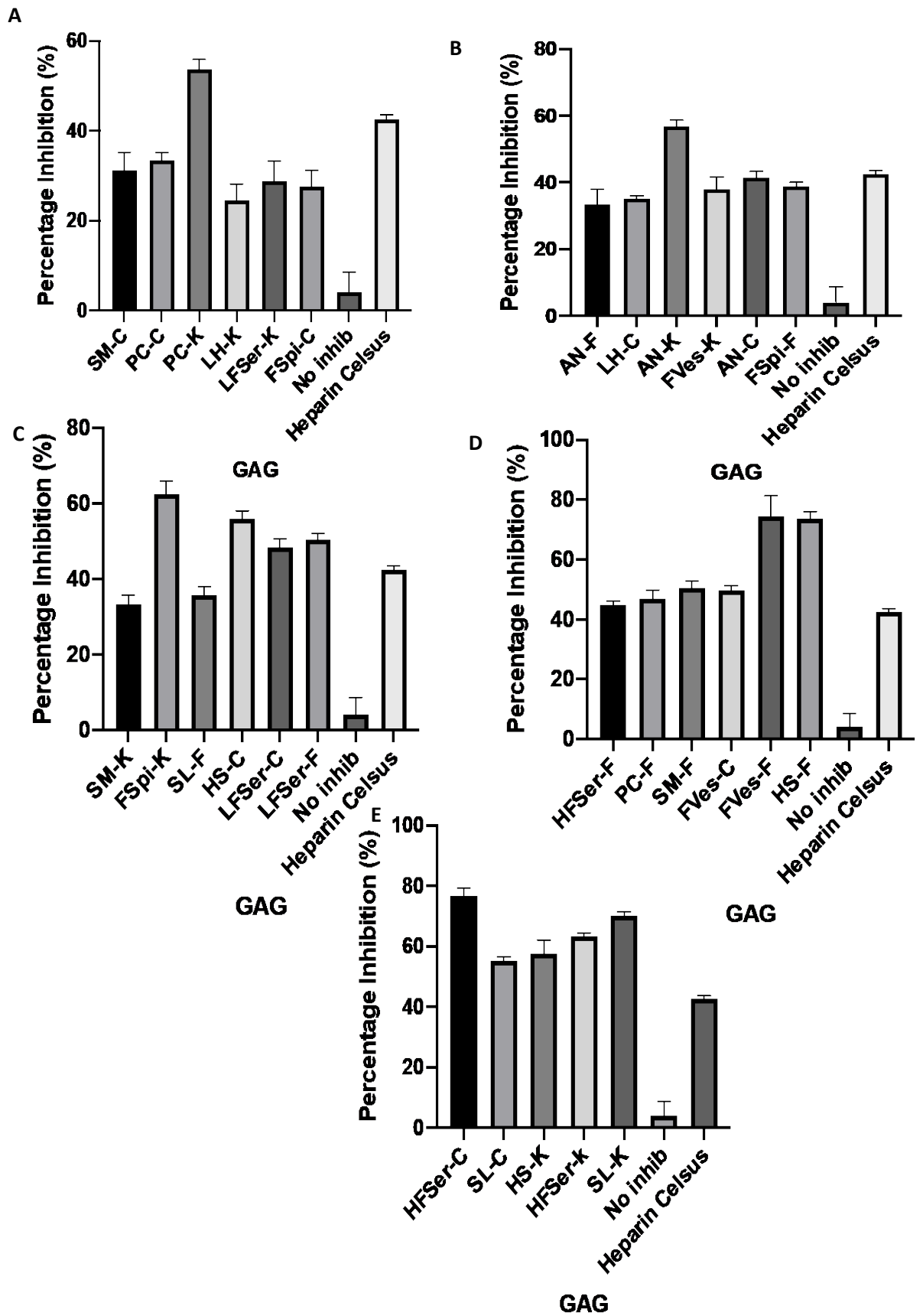tes, the plant compounds with the most inhibitory activity were SL-K, FVes-F and HSer-C, with percentage inhibitions of 72%, 75% and 78% respectively.

The inhibitory activity of heparin and 29 various plant derived sulphated polysaccharides was examined for potential inhibition of RBD and ACE2 binding. Inhibition was measured in vitro using an ELISA with a specific Rabbit anti-RBD antibody (Stratech). 3,3',5,5'-Tetramethylbenzidine, a chromogen that when oxidised yields a blue colour, as a result of oxygen radicals produced by the hydrolysis of hydrogen peroxide by HRP was utilised. The colour then changes to yellow following the addition of sulphuric acid, the absorbance was measured at a wavelength of 450nm.

Heparin showed percentage inhibition of 42%. All compounds decreased the binding activity of RBD to ACE2, displaying inhibitory activity. Of the 29 investigated compounds, 17 showed greater inhibitory activity than heparin, in ascending order, HFSer-F (44%), LFSer-C (48%), PC-F (48%), FVes-C (49%), SM-F (50%), LFSerF (52%), PC-K (54%), SL-C (55%), HS-C (56%), AN-K (58%), HFSer-K (58%), FSpi-K (63%), HS-F (72%), SL-K (72%), FVes-F (75%) and HSer-C (78%).

4.2. Summary of results:

Preliminary research was aimed at determining the optimal dye for use in differential scanning fluorimetry assays, two dyes SYPRO Orange and ProteOrange- the more affordable alternative, were assessed. Two conditions were investigated, the concentration of fluorescent dye used, and type of protein used in DSF experiments. These investigations revealed that SYPRO Orange, although more expensive was the optimal dye for use in differential scanning fluorimetry. The optimal concentration of SYPRO Orange with both fibrinogen and lysozyme was investigated. Across all investigations, the optimal concentration of protein for use in DSF was determined to be 400µg/mL (Figures 11-14). However, the concentration of fluorescent dyes, SYPRO Orange and ProteOrange varied depending on the protein utilised and the concentration of proteins. With regards to lysozyme, SYPRO Orange was found to be the optimal dye at lower concentrations from 25X upwards, Figure 15, in contrast, fibrinogen, Figure 16, the opposite was seen, with ProteOrange being the most optimal from 25X upwards. Due to the scarcity of proteins at the time of writing, these preliminary experiments were not applied to the following experiments but provide a basis of research which may assist in fluorescent dye choices for optimal DSF assays.

This data confirms published data that demonstrates that heparin does not only bind to SARS-CoV-2 RBD but also destabilises the secondary structure of the receptor binding domain. In both mutations of SARS-CoV-2 utilised, heparin destabilises the secondary structure to produce a T_m shift of at least 2°C. More significantly in the UK SARS-CoV-2 mutation, Figure 18, shows that there was a 2.59°C shift in T_m compared to 2°C in the MN908947 mutation, Figure 19. The MN908947 mutation SARS-CoV-2 S1 protein in the presence of heparin had a lower fluorescence emission when compared to bound SARS-CoV-2 S1, by 300RFU, which could indicate that a larger unfolding event occurs when the S1 domain is alone in solution than when bound to heparin.

ELISA assays investigated the inhibitory activity of both heparin and a library of sulphated plant compounds on the binding of SARS-CoV-2 to ACE2 receptors. Heparin was found to inhibit binding of the SARS-CoV-2 RBD to ACE2 by 42%, Figure 20. 29 sulphated plant compounds were found to also decrease the binding SARS-CoV-2 RBD to ACE2 receptors, 17 of which were more inhibitory than heparin. In ascending order, HFSer-F (44%), LFSer-C (48%), PC-F (48%), FVes-C

(49%), SM-F (50%), LFSerF (52%), PC-K (54%), SL-C (55%), HS-C (56%), AN-K (58%), HFSer-K (58%), FSpi-K (63%), HS-F (72%), SL-K (72%), FVes-F (75%) and HSer-C (78%).

These results aimed to determine heparin or sulphated plant compound binding to SARS-CoV-2, using differential scanning fluorimetry and ELISA. However, due to SARS-COV-2 restrictions on the laboratory use, these results have been limited. Further explorations would need to be carried out to elucidate the interaction between these compounds and SARS-CoV-2 RBD, such as structural investigations like Circular Dichroism and Surface Plasmon Resonance studies. These would confirm whether the binding altered the conformation of the protein to confirm the potential for therapeutic use. Further studies like viral plaque or titre assays could have been carried out to determine effective and inhibitory concentrations of heparin and plant compounds. Moreover, different forms of heparin, like LMWH could have been investigated as they more favoured in a clinical environment.

Discussion:

The rapid and ongoing spread of SARS-CoV-2 initiated a call to action for the investigation into fast, accessible and safe treatments. It was proposed that pre-approved pharmaceuticals be repurposed in order to avoid the arduous and time-consuming process of full clinical trials. Heparin has been used safely for a number of decades as an adjunct therapy for surgery, during dialysis and during blood transfusions, with a primary modality in the treatment of blood clots. Previous research suggests that heparin, a common anticoagulant, can be used to treat a number of viral infections, with a large body of evidence supporting its use in coronaviruses.

Differential scanning fluorimetry was used to assess the interactions of various proteins, fluorescent dyes and sulphated polysaccharides. DSF is an affordable, accessible, and fast method for monitoring the refolding of a protein as it progressively denatures. This relies on the fluorescence of dyes as they interact with proteins in their denaturation transition between a strongly hydrated solvated state and the final aggregated solid state. Denaturation is controlled by slow heating of the protein solution from a base-line, natural temperature, in this case 25°C. The heating induces changes in the water shells and the hydrogen bonds, leading to associated changes in the protein conformation. Specifically designed dyes interact with the bonds, this interaction is signalled by changes in the fluorescence spectrum. To determine the optimal dye for recording the thermal denaturation of SARS-CoV2-RBD in the presence of heparin, preliminary experiments using lysozyme and fibrinogen with the dyes SYPRO Orange and its analogue ProteOrange. SYPRO Orange has a slightly wider range of detection 4-8ng of protein compared to ProteOrange, 3ng of protein per band. ProteOrange is 10X more sensitive than Coomassie, but less sensitive than silver staining, whereas, SYPRO Orange has similar sensitivity to silver staining but is more similar than

Coomassie. Following substantial analysis, it was determined that the optimal dye for analysis was SYPRO Orange, given that it can be used at lower concentration whilst yielding higher or similar fluorescence emission values than ProteOrange at the same concentration. As shown in Figure 15, where the emission of SYPRO Orange and ProteOrange in the presence of lysozyme is compared. At a concentration of 50x, ProteOrange produced the highest fluorescence emission, ~12500RFU, compared to SYPRO Orange, ~6000RFU. When the concentration was halved, 25x, SYPRO Orange emitted the same emission, ~12500RFU, compared to a lower emission, ~8000RFU with ProteOrange. ProteOrange is a valuable analogue, that in the presence of fibrinogen emits a higher fluorescence, as seen in Figure 14, ProteOrange at a concentration of 25x had a larger emission of fluorescence than SYPRO Orange, ~2700RFU compared to ~1000RFU. This analysis determined that the optimal dye for use in following experiments, as a result of its ability to be used at a lower concentration and higher emission of fluorescence.

Recently, cell surface heparan sulphate proteoglycans were implicated as co-receptors for SARS-CoV-2 spike protein and facilitate subsequent binding to ACE2 receptor. To further investigate this, the binding activity of heparin, a proxy for heparan sulphate, and SARS-CoV-2 receptor binding domain was probed. The thermal stability of the RBD in the presence of heparin was assessed utilising SYPRO Orange at a concentration of 25x. The presence of heparin elicited a notable reduction in the melting temperature of 2.5°C with the recombinant RBD, similarly, with SARS-CoV-2 spike S1 RBD a reduction of 2°C was seen in the presence of heparin. This agrees with data that demonstrates a reduction of 3.25°C using Celsus heparin and exploratory data on Wockhardt heparin, that indicated a similar reduction in melting temperature of 2.4°C (Tree, *et al*., 2020). This demonstrates a high affinity for heparin binding *in vitro* with consequences on the thermal stability of the protein, which indicates possible adjustments to protein structure. These changes in protein conformation may have broader implications *in vivo*, that will need to be explored thoroughly through viral plaque and binding assays.

The application of heparin as a first line response to treatment in the case of coronavirus has been limited by its potent anticoagulant activity. With the risk of coagulopathies already extremely high in clinical cases, it is not recommended to put patients further at risk by treating them in this manner, despite promising evidence that it binds and affects the structure of the binding domain and may improve clinical outcomes significantly. Importantly, pharmaceutical grade heparin remains a heterogenous polydisperse mixture of natural products with both anticoagulant and non-anticoagulant saccharide structures. These anticoagulant fractions may become more useful for the future of antiviral agents that have little to no anticoagulant effects whilst still retaining biological activity. However, investigations into the processes for the extirpation of anticoagulant properties by enzyme or chemical treatment are currently ongoing.

Given the anticoagulant properties of heparin, sulphated synthetic and naturally derived polysaccharides that act in a similar manner have emerged as an area of interest. The potential of sulphated polysaccharides as SARS-Cov-2 binding inhibitors has only been briefly touched on using the ELISA. A large proportion of the plant compounds screened showed inhibitory activity equal to or even exceeding the inhibitory activity of heparin. Sulphated polysaccharides with potential include HS-F, SL-K, FVes-F and HSer-C, with inhibitory activity from 72-78%, compared to heparin: 42%. This provides a basis for which further investigations can be carried out, perhaps using differential scanning fluorimetry to determine the effect of plant polysaccharide binding on stability of the RBD. Once efficacy is determined, it would be advantageous to utilise these polysaccharides in cell lines, carrying out viral plaque and binding assays. Despite promising results, the likelihood of these compounds being used as a treatment is very low, due to the fact that they are unlicensed. However, these results provide further evidence to bolster the idea that polysaccharides can have therapeutic effects, whilst avoiding the adverse and off target effects that heparin causes.

Experimental data provides preliminary evidence for the further investigation of heparin and sulphated carbohydrates and SARS-CoV-2, with the aim to elucidate the binding relationship to provide a basis for potential therapeutic treatment. This agrees with the existing data with a great deal of data supporting the use of heparin, heparin mimetics and low molecular weight heparins as a clinical treatment for SARS-COV-2. This is ultimately beneficial, with concerns regarding vaccine escape and the rapid mutation of SARS-CoV-2 resulting in the need for therapeutic treatment that circumvent the typically targeted antiviral pathways. Further, drug repurposing provides a time and cost-effective area of research, expediting the approval process and clinical implication if treatment proves successful.

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Appendix:

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