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Invited Article

Therapeutic antibodies – natural and pathological barriers and strategies to overcome them

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ABSTRACT

Antibody-based therapeutics have become a major class of therapeutics with over 120 recombinant antibodies approved or under review in the EU or US. This therapeutic class has experienced a remarkable expansion with an expected acceleration in 2021–2022 due to the extraordinary global response to SARS-CoV2 pandemic and the public disclosure of over a hundred anti-SARS-CoV2 antibodies. Mainly delivered intravenously, alternative delivery routes have emerged to improve antibody therapeutic index and patient comfort. A major hurdle for antibody delivery and efficacy as well as the development of alternative administration routes, is to understand the different natural and pathological barriers that antibodies face as soon as they enter the body up to the moment they bind to their target antigen. In this review, we discuss the well-known and more under-investigated extracellular and cellular barriers faced by antibodies. We also discuss some of the strategies developed in the recent years to overcome these barriers and increase antibody delivery to its site of action. A better understanding of the biological barriers that antibodies have to face will allow the optimization of antibody delivery near its target. This opens the way to the development of improved therapy with less systemic side effects and increased patients' adherence to the treatment.

Contents

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; ADC, antibody-drug conjugate; BBB, blood-brain barrier; CNS, central nervous system; ECM, extracellular matrix; EU, European union; Fab, fragment antigen-binding; Fc, fragment crystallizable; FcRn, neonatal Fc receptor; FDA, Food and Drug Administration; GI, gastrointestinal; HER2, human epidermal growth factor receptor 2; IA, intra-articular; IdeS, bacteria secreted proteases; IFP, interstitial fluid pressure; IgG, immunoglobulin G; IVIg, intravenous immunoglobulin; mAb, monoclonal antibody; MMP, matrix metalloproteinase; PD, pharmacodynamics; pI, isoelectric point; PK, pharmacokinetic; scFv, single-chain variable fragment; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor; US, United States.

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1. Introduction

Today, recombinant antibodies represent an important therapeutic class in the armamentarium of clinicians, with some products standing as first-line standard of care. Recombinant antibodies exist in diverse forms, whether they are monoclonal or multivalent, full-length IgG or Ab fragments, first-in-class molecule or biosimilar, naked or conjugated to cytotoxic agents (antibody drug conjugate; ADC) (Elshiaty, Schindler, & Christopoulos, 2021). In fact, there are more than 120 molecules approved or in review in the EU and US, most of them being full-length unconjugated mAb from the IgG1, IgG2 and IgG4 subclasses (Vukovic, van Elsas, Verbeek, & Zaiss, 2021). Since 2014, six to twelve new recombinant antibodies are granted first approval (in either the US or EU) each year (https://www.antibodysociety.org/resources/approved-antibodies/). Recombinant antibodies constitute a thriving focus of research in oncology and non-cancer diseases (autoimmune, infectious diseases, ophthalmic, dermatologic, respiratory disorders), as they have already proven their efficiency in a variety of diseases, ranging from small patient populations with orphan diseases to hundreds of thousands of patients. The success of recombinant antibodies relies on: (i) a unique and complex pharmacokinetic profile, with full-length IgG exhibiting long half-lives, (ii) a multifaceted pharmacodynamics (PD) with multiple modes of action, and (iii) a good safety profile, generally well tolerated and with a low risk of unexpected side effects in humans. These qualities often provide a first-to-market advantage, as antibodies can move rapidly from proof-of-concept studies towards commercialization.

The therapeutic response to antibodies relies on their interdependent PK and PD, with PK influencing the magnitude and duration of antibody PD, and PK being influenced by the biology of the target antigen, a concept known as target-mediated drug disposition. PK is characterized by antibody absorption, distribution, metabolism and elimination (Kamath, 2016). Antibody absorption depends mainly on the route of administration. While intravenous injection remains the most commonly used administration route for Ab, there has been an increasing trend towards the development of alternative delivery modalities to treat patients with chronic diseases. This should enable self-administration or caregiver-supported dosing at home in order to reduce hospital/clinical cost while improving patients' quality of life and treatment (Sanchez-Felix, Burke, Chen, Patterson, & Mittal, 2020). It should also improve antibody therapeutic index when the intravenous injection is not adapted to achieve high Ab concentration at the site of action and/or results in undesirable on-target/off-tissue side effects. Since 2009, the subcutaneous route has shown a marked interest. With a third antibody approved in the US, this route is the predominant alternative delivery route, and commonly used to treat immunological chronic diseases, such as asthma, rheumatoid arthritis and psoriasis (Matucci, Vultaggio, & Danesi, 2018). Other administration routes of marketed Ab include intravitreal for anti-vascular endothelial growth factor (VEGF) in ophthalmic disorders, inhalation, oral and intra-articular delivery which constitute a growing focus of research for local-acting antibodies, with several molecules being evaluated in clinical trials (inhalation: NCT04631705, NCT01818024; oral: NCT01205087, NCT01459419; intra-articular: NCT00819572, NCT04731675, NCT04814368). The distribution and subsequent binding of the Ab to its target antigen depends on its partitioning properties in the different body compartments, and are directly impacted by the antibody format, the expression/concentration of the target antigen, the route of administration and biological barriers associated to the physio-pathological conditions. Metabolism of intravenous IgG mainly occurs in the vascular endothelium, following saturation of the FcRn receptor, which is known to rescue IgG Ab from intracellular catabolism. Once in the tissue, antibodies catabolism may take place after interaction with the target antigen (target-mediated drug disposition) or by non-specific mechanisms (Glassman, Abuqayyas, & Balthasar, 2015).

Here, we comprehensively review the different biological barriers that recombinant antibodies must overcome to access their target antigen, associated either to physiological or pathological conditions and encountered depending on the route of administration. Understanding the influence of these biological barriers on the kinetics and partitioning of antibody distribution is necessary for accurate predictions of antibody PK and ultimately therapeutic response. In addition, we describe strategies recently developed to overcome these barriers and improve the efficacy of antibody-based therapeutics.

2. Extracellular barriers

2.1. Extracellular matrix

2.1.1. Structure and functions of the extracellular matrix

In normal and malignant tissues, the extracellular matrix (ECM) is defined as a non-cellular component that provides biochemical and structural support for its cellular constituents (Walker, Mojares, & Del Rio Hernandez, 2018) (Fig. 1). Two main ECM structures can be found: (i) the basement membrane, a very compact ECM found in both epithelial and endothelial tissues (Kruegel & Miosge, 2010); and (ii) the interstitial matrix, a looser matrix found below the basement membrane as well as between connective tissues. Structurally, the ECM core components are collagens, fibronectins, laminins, proteoglycans, hyaluronan, and other (glyco)proteins such as matricellular proteins (Theocharis, Manou, & Karamanos, 2019). Collagens are the most abundant structural proteins of the ECM and are produced by fibroblasts, endothelial and epithelial cells. They are found mainly in the ECM of connective tissues, providing tensile strength. Collagens have been shown to be involved in cell adhesion and migration. Fibronectins are located within the basement membrane and arranged into a mesh of fibrils similar to collagens. They are linked to cell surface receptors (integrins) and play a key role in cell adhesion, embryonic development and wound healing processes. Laminins are important components of the basement membrane that create a network with collagens by bridging molecules such as perlecan. They interact with epithelial cells via surface cell receptors such as integrins or cell surface proteoglycans. Laminins are involved in cell adhesion and play a pivotal role in cell differentiation and migration. Proteoglycans are core proteins onto which a few to hundreds of glycosaminoglycans (e.g., hyaluronan, heparan sulphate) side chains are linked. They are scattered among collagen fibrils. Proteoglycans sequester water within tissues, conferring to them hydration functions. Glycosaminoglycans also bind many growth factors, sequestering them in the ECM. Matricellular proteins are non-structural proteins that encompass thrombospondins, tenascins, fibulins, osteonectin, osteopontin, or periostin. Secreted in the ECM, they can interact with matrix proteins, growth factors and several cell surface receptors. They play a major role in cell-matrix communication. These components are organised in a structural meshwork that interacts with host cells through surface receptors including integrins, discoidin domain receptors, cell surface proteoglycans, or hyaluronic acid receptors such as CD44. The ECM does not only supply cells with a physical structure, but also provides chemical and mechanical signals known to regulate migration, proliferation, survival, as well as differentiation to maintain homeostasis (Koohestani et al., 2013; Pickering, 2001; Quaranta, 2000). The ECM is a highly dynamic structure that goes through continuous remodelling under normal (e.g., wound healing) or pathological conditions. ECM originates from a broad range of cells, but fibroblasts are the major source of ECM during remodelling (Bonnans, Chou, & Werb, 2014). Notably, ECM remodelling occurs after matrix degradation and is critical for the regulation of ECM abundance, composition, structure, as well as the release of biologically active molecules such as growth factors. Matrix-degrading enzymes include matrix metalloproteinases (MMPs), adamalysins (e.g., a-disintegrin and metalloproteinases, ADAMs; and ADAMs with thrombospondin motifs, ADAMTS), meprins, plasminogen activators, and extracellular proteases such as serine proteases, cathepsins and

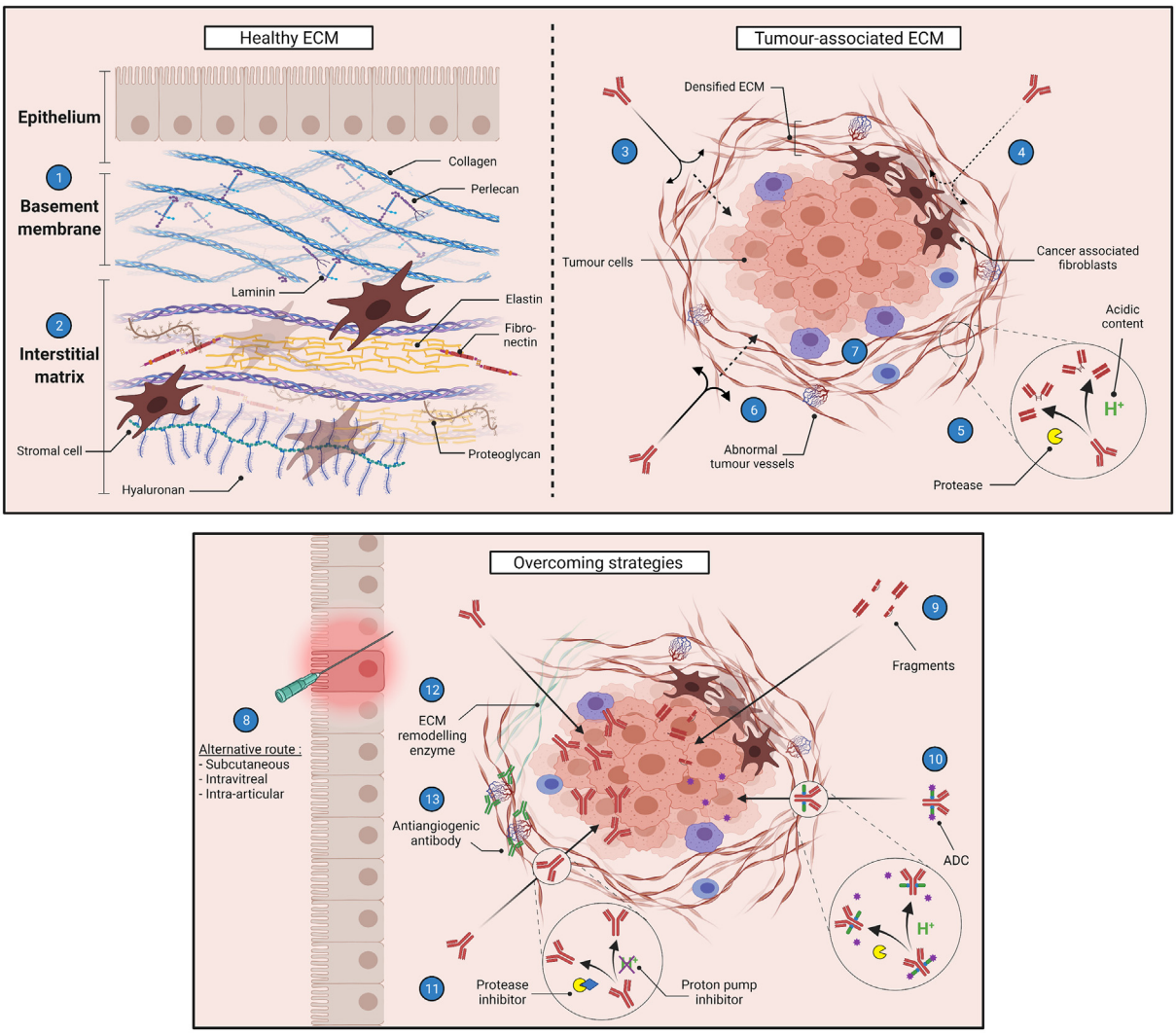

Fig. 1. Schematic representation of the extracellular matrix in healthy condition and in the tumour microenvironment as a barrier to antibodies.
A healthy ECM (left panel), located under the epithelium, consists in two distinct structures. The basement membrane (1) is composed of a very compact network of collagen fibrils and laminins that can self-associate to form a secondary network; perlecan further bridge these networks. The interstitial matrix is a looser matrix mainly composed of collagen fibrils assembled through the participation of fibronectin. Other components, including elastin, proteoglycans, and hyaluronan, contribute to the ECM organisation. Stromal cells present in the interstitial matrix interact with ECM components and growth factors. In cancers (right panel), tumour cells and cancer-associated fibroblast secrete a plethora of ECM proteins (such as collagens, fibronectin, elastin, or laminins), proteoglycans, cytokines, and growth factors. This ECM differs significantly in conformation and composition from that of normal tissues. The excessive accumulation of dense and rigid ECM results in the encapsulation of tumour cell clusters, acting as a physical barrier for antibody diffusion (3) (4). The acidic microenvironment associated with increased concentration of proteases within the ECM (5) represents a biochemical barrier degrading antibodies and reducing ADC efficacy. The increase interstitial pressure contributes to repelling antibodies (6). Finally, ECM-mediated hypoxia and low immune cell infiltrate (7) reduces antibody effectiveness and especially immunotherapy treatments. Strategies to overcome the ECM barrier (lower panel) and increase antibodies distribution and diffusion in the vicinity of the mAb target have been developed. Alternative administration routes have been considered (e.g. subcutaneous, intravitreal, or intra-articular) (8). Antibody fragments exhibit higher diffusion rate through the stroma as compared to full length antibodies (9). ADCs have been specifically designed to manipulate ECM acidic pH and high protease concentration for the efficient release of their payload (10). Combination of mAb with pH pump inhibitors or proteases inhibitors can also be considered to lower ECM-associated biochemical barrier (11). ECM remodelling strategies have been evaluated to increase antibodies diffusion and therapeutic efficacy (12). Finally, antiangiogenic mAbs can be used to restore interstitial fluid pressure and normalize anarchic blood vessels growth in order to enhance antibody delivery (13).

granzymes (Bonnans et al., 2014) (see Section 2.2). Non-proteolytic enzymes involved in ECM remodelling include hyaluronidases and heparanase (Bonnans et al., 2014). It is noteworthy that aberrant ECM remodelling is at the origin of various pathologies. For example, abnormally high ECM breakdown is observed in cardiomyopathy or osteoarthritis (Maldonado & Nam, 2013; Silva, Pereira, Fonseca, Pinto-do, & Nascimento, 2020). On the contrary, excessive ECM production and deposition without balanced degradation can lead to fibrosis in the context of chronic or severe tissue injuries (Herrera, Henke, & Bitterman, 2018). Finally, tumorigenesis is highly associated with alteration of ECM structure, composition, and remodelling.

2.1.2. The ECM as a barrier for antibody delivery and therapeutic efficacy in the context of cancer

In cancers, the ECM differs significantly from that of normal tissues as well as between molecular tumours subtypes (Fig. 1). Many solid tumours demonstrate high expression of ECM proteins such as collagens, fibronectin, elastin or laminins. Moreover, a hyaluronic acid-rich ECM characterizes few cancer types, such as pancreatic ductal adenocarcinoma (Hessmann et al., 2020). The ECM is a major component of the tumour microenvironment, accounting for up to 60% of the tumour mass. Tumour cells are themselves the source of ECM, and to an even greater extend cancer-associated fibroblasts. This desmoplastic reaction is

linked to poor patient prognosis and described as a major driver of cancer therapy resistance.

The excessive accumulation of dense and rigid ECM results in the encapsulation of tumour cell clusters, acting as a physical barrier for the diffusion of therapeutic agents. The role of ECM composition and organization on IgG penetration was assessed *in vivo* in xenograft tumour models (Netti, Berk, Swartz, Grodzinsky, & Jain, 2000). Netti et al. also showed that resistance to IgG penetration is related to tumour rigidity and collagen organization. An inverse correlation between sulphated glycosaminoglycans and IgG penetration was later reported (Davies Cde, Berk, Pluen, & Jain, 2002). 3D *in vitro* co-culture cancer models were recently developed to assess IgG distribution within the ECM (Estrada et al., 2016; Rebelo et al., 2018) and a computational (*in silico*) model was calibrated based on these data (Cartaxo et al., 2020). Such models offer a valuable and modular tool to study the influence of different components of the ECM on antibody transport without the need for *in vivo* models. The ECM barrier impairs the diffusion of oxygen, leading to highly hypoxic regions in the tumour mass. Histological analysis of tumour samples from patients highlighted that hypoxia results in increased collagen deposition and fibrosis in hypoxic regions (Shekhar, Pauley, & Heppner, 2003). The low microvessel density observed in tumours with high ECM deposition further aggravates hypoxia (Edgar, Underwood, Guilkey, Hoying, & Weiss, 2014). This hypoxia factor was used for the activation of prodrugs (Zhou et al., 2019) and the cleavage of ADC linkers (Staudacher & Brown, 2017), but can also confer resistance to ADCs. This was recently showed for trastuzumab-emtansine (T-DM1) in HER2+ breast cancer cells. Indeed, Chandran et al. demonstrated that trastuzumab internalization was reduced in hypoxic conditions due to a redistribution of phosphorylated caveolin-1 from vesicular membrane raft to the plasma membrane (Indira Chandran et al., 2020). Hypoxia is also linked to radiotherapy resistance through different mechanisms such as reduced production of oxygen radical species, reduced fixation of oxygen on DNA radical preventing its repair, or production of hypoxia-inducible factor (HIF-1) that will increase the production of antioxidant and thus buffer radiation-induced reactive oxygen species (Pouget, Georgakilas, & Ravanat, 2018; Pouget, Lozza, Deshayes, Boudousq, & Navarro-Teulon, 2015). Therefore, combating tumour hypoxia is considered as an approach to improve radioimmunotherapy effect (Boreel, Span, Heskamp, Adema, & Bussink, 2021).

Cancer immunotherapy using inhibitory Ab against the cytotoxic T-lymphocyte-associated protein 4 (CTLA4), and the programmed cell death ligand and protein 1 (PD-L1/PD-1) attracted a lot of attention due to the outstanding therapeutic response observed in patients with melanoma or non-small cell lung cancer. However, most cancers remain refractory to this approach. A major limitation of immunotherapy approaches is the need to get both drug and T cells through the ECM to reach tumour cells in order to be effective. Immune cells attracted by tumour-induced cytokine gradient are redirected when confronted with the rigid ECM that surrounds tumour cells clusters. Upon contact with the rigid ECM, lymphocytes will migrate along the fields of elevated rigidity (haptotaxis) (Hallmann et al., 2015). Salmon et al. demonstrated that in human lung tumour samples, the distribution of T cells is dictated by the aligned collagen fibres surrounding tumour islets (Salmon et al., 2012). Therefore, the reduced diffusion of antibody and immune cells throughout the ECM highly contributes to the low response of ECM-rich tumours to immunotherapy treatment.

2.1.2.1. Delivery through cancer ECM: overcoming strategies. Tumour ECM ostensibly contributes to transport resistance, and diffusion coefficient correlates inversely to the molecular hydrodynamic radius of any therapeutics (Xenaki, Oliveira, & van Bergen En Henegouwen, 2017; Yokota, Milenic, Whitlow, & Schlom, 1992). Diffusivity assays with dextrans of molecular weights ranging from 4 to 70 kDa showed a decrease of diffusion throughout the ECM for molecules with high molecular weight (Galgoczy et al., 2014). One could extrapolate this conclusion and hypothesise that antibody fragments should better diffuse in the ECM as compared to full-length IgG, however, there is a current lack of comparative studies to prove this hypothesis. Remodelling the extracellular matrix appears also as an evident strategy to improve the therapeutic efficacy of full-length antibody, ADC or radio-immunoconjugates. Eikenes et al. showed that collagenase treatment in a human osteosarcoma xenograft model resulted in a 2-fold increase in tumour uptake and improved distribution of a specific mAb (Eikenes, Bruland, Brekken, & Davies Cde, 2004). Depletion of hyaluronic acid using a pegylated recombinant human PH20 (PEGPH20) resulted in an increase in natural killer cell access to high-hyaluronic tumour cells, and enhanced antibody-dependent cellular cytotoxicity (ADCC) effect of both trastuzumab and cetuximab *in vitro* (Singha et al., 2015). This approach was translated *in vivo* and resulted in the further inhibition of tumour growth in tumour-bearing mice upon combined treatment of trastuzumab, PEGPH20 and natural killer cells when compared to control groups. Depletion of hyaluronic acid was further assessed for the unmasking of the HER2. Trastuzumab-resistant JIMT1 breast cancer cells were treated with hyaluronidase and 4-methylumbelliferone Degradation of hyaluronan and inhibition of its synthesis were achieved by treating trastuzumab-resistant JIMT1 breast cancer cells with hyaluronidase and 4-methylumbelliferone to degrade hyaluronan and inhibit its synthesis. This combined treatment resulted in the increased uptake of a radiolabelled trastuzumab conjugate ([^{89}Zr]Zr-trastuzumab) and the enhanced efficacy of trastuzumab in JIMT1 xenografts (Pereira et al., 2020).

Numerous ECM targets have been evaluated for the development of therapeutic antibodies, and especially ADCs, with the aim of overcoming ECM pitfalls by exploiting its specific features for the efficient release of the payload. Matsumura and his team described an innovative ADC that targets specifically cancer-associated fibrin using a chimeric mAb (Matsumura, 2012; Yasunaga, Manabe, & Matsumura, 2011; Yasunaga, Manabe, Tarin, & Matsumura, 2011). This chimeric ADC, targeting ECM fibrin, comprised the conjugation of the active metabolite of irinotecan (SN-38) through an acid-labile linker (ester), sensitive to the acidic tumoral microenvironment. This results in a potent antitumor activity by damaging both cancer cells and tumour vasculature (M. Yasunaga, Manabe, & Matsumura, 2011). In a comparable approach, the same group designed a similar ADC targeting collagen IV in the ECM (Yasunaga, Manabe, & Matsumura, 2013). Non-structural ECM proteins, such as Tenascin-C or fibronectin, overexpressed in various human cancers (e.g., epidermoid carcinoma, glioblastoma, non-small cell lung cancer, head and neck cancers and triple negative breast cancers), were further investigated for ADC development. A site-specific ADC was produced from a mAb targeting a splice form of tenascin C, conjugated with monomethyl auristatin E (MMAE) through a protease-sensitive linker. This linker is cleaved by proteases such as cathepsin B, which activity is increased in the tumour microenvironment. Furthermore, the acidic tumour microenvironment may facilitate the extracellular activation of these hydrolytic enzymes. Complete tumour regression was observed in 3 out of 5 mice after 4 administrations of the ADC (7 mg/kg every 3 days) in A431 human epidermoid carcinoma xenografted-BALB/c nude mice (Dal Corso, Cazzamalli, Gebleux, Mattarella, & Neri, 2017). A smaller ADC based on a small immune protein, consisting of a scFv fragment fused to the human IgE εCH4 domain, was developed to target the alternatively spliced Extra Domain A (EDA) of fibronectin (Casi & Neri, 2012, 2015; Gebleux, Wulhfard, Casi, & Neri, 2015; Perrino et al., 2014). This small immune protein was conjugated to two emtansine (DM1) molecules that can be cleaved by the high level of extracellular glutathione in the tumour microenvironment. The resulting ADC led to total remission of tumour mass in 4 out of 5 mice (5 injections at 5 mg/kg), whereas no effect was observed with the control IgG drug conjugate. In the context of refractory and ECM-rich cancers, these studies highlight that the ECM diffusion barrier can be overcomed by targeting ECM non-internalising targets and taking advantage of ECM extracellular stimuli for a sustained ADC payload

release (Joubert, Beck, Dumontet, & Denevault-Sabourin, 2020; Joubert, Denevault-Sabourin, Bryden, & Viaud-Massuard, 2017).

2.1.3. ECM and administration of antibody through the skin

The skin is composed of an outer avascular layer – the epidermis – a core connective tissue layer – the dermis – and finally a subcutaneous layer – the hypodermis (Arda, Goksugur, & Tuzun, 2014). Antibodies exhibit low skin permeability when applied topically (Bruno, Miller, & Lim, 2013). It may be explained by the hydrophobic nature of the keratin, contrasting with the hydrophilic and polar features of antibodies, limiting their diffusivity through the skin. In addition, antibodies exhibit low cellular and transcellular diffusion capabilities. Thus, high doses of antibody may be required by the trans-appendageal route to achieve local therapeutic response. Additional hurdle comprises the nature of products for topical delivery – gel or cream are often intended for topical skin application –, which will require complex formulation engineering to maintain antibodies stability (Stevens & Cowin, 2017). Overall, antibody treatment of lesions with intact skin epithelium remains challenging. New technologies investigate cell-penetrating peptides, physical penetration enhancer or injection with microneedles to help antibody cross the skin barrier (Francis & Thomas, 2017; Gautam et al., 2016; Geh et al., 2019; Lee, Park, & Kim, 2018; Oberli, Schoellhammer, Langer, & Blankschtein, 2014), but none of them already achieved clinical success.

Subcutaneous injection of antibody is particularly attractive, as it has been shown to be safe and valuable for both patients and healthcare workers (Sanchez-Felix et al., 2020). Currently, at least one-third of FDA-approved recombinant antibodies are administered by subcutaneous injection and therefore face this barrier for Ab distribution and efficacy (Viola et al., 2018). The subcutaneous injection of antibodies is predominantly used in immunology for the treatment of chronic diseases such as rheumatoid arthritis or plaque psoriasis, targeting interleukins and their receptors, thus the subcutaneous route is mostly intended for antibody with a systemic action (Sanchez-Felix et al., 2020). When considering a subcutaneous administration, the formulated drug is delivered into the hypodermis interstitial space. The hypodermis is composed of adipose tissue, unevenly distributed blood and lymph vessels, fibroblasts, macrophages and an ECM network rich in collagen, elastin and glycosaminoglycans (Arda et al., 2014). In this context, the ECM constitutes the first physical barrier for subcutaneous delivery, as Ab must pass the ECM to reach the vascular compartment (Viola et al., 2018). Collagen fibrils are positively charged at physiological pH while hyaluronic acid and chondroitin sulfate, two major glycosaminoglycans of the hypodermis ECM, are negatively charged. Despite collagen and glycosaminoglycans having opposite charges, the hypodermis interstitial space is negatively charged due to the presence of negatively charged proteoglycans, rendering the transport of negatively charged drug more rapid thanks to electrostatic repulsion (Viola et al., 2018). One should note that most mAb have an isoelectric point (pI) between 7 and 9, meaning that mAbs are generally positively charged at physiological pH. Bumbaca Yadav et al. evaluated the impact of the variable region charge (+3/+5 versus −4) on mAb subcutaneous bioavailability in Cynomolgus monkeys. They reported that positively charged mAb present a reduced subcutaneous bioavailability by 31%, while their negatively charged counterpart demonstrates enhanced subcutaneous bioavailability up to 70% (Bumbaca Yadav et al., 2015). Furthermore, positively charged mAb (pI = 7.3; 9.1) showed electrostatic interaction with negatively charged ECM components in another study, leading to 50–60% unrecoverable/tissue bound mAb. Co-injection with hyaluronic acid resulted in the recovery of 70% of soluble Ab, highlighting that hyaluronic acid does compete with mAb binding sites and that such formulation strategy enhances the subcutaneous bioavailability of positively charged mAb (Mach et al., 2011). The ECM negative charges are also responsible for interaction with water molecules and consequently impacts the ECM hydraulic conductivity and interstitial fluid. Because of the latter, subcutaneous injection volume is limited to 2 mL in humans.

2.1.3.1. Subcutaneous delivery: overcoming strategies. To facilitate subcutaneous bulk fluid flow and increase the dispersion and absorption of drugs, a recombinant human hyaluronidase PH20 (rHuPH20) was used to locally and transiently degrade hyaluronan (Locke, Maneval, & LaBarre, 2019). Clinical studies confirmed the potential of this approach to improve the pharmacokinetic profiles of co-administered mAb (e.g. rituximab, trastuzumab, cetuximab) compared to subcutaneous injection without rHuPH20 (Printz et al., 2020; Shpilberg & Jackisch, 2013; Styles et al., 2019). Another study achieved a comparable body exposure to trastuzumab after subcutaneous injection at 8 mg/kg as compared to intravenous injection at 6 mg/kg (Wynne et al., 2013). These studies led to the FDA approvals of rHuPH20 in combination with rituximab in 2017, trastuzumab in 2019 (Locke et al., 2019), and trastuzumab plus pertuzumab in 2021 (Tan et al., 2021). The hypodermis interstitial fluid is mostly composed of plasma water with only about 50% of its protein content. Among these proteins, proteases play a major role in antibody catabolism and therefore reduces antibody subcutaneous bioavailability (see Section 2.2). Once in the hypodermis interstitial space, antibody will be absorbed by convection into lymphatic vessels as their large size prevents them from diffusing directly into the bloodstream. Such knowledge further underlines the relevance of subcutaneous injection for antibody targeting the lymphatic system like interferons or interleukins.

2.1.4. ECM and retinal/eyes delivery of antibody

The prevalence of age-related posterior eye diseases, which most frequent example being age-related macular degeneration, will reach several hundred billion in the next decades (Wong et al., 2014). The delivery of drugs to targets in the retina is challenging and various routes of administration have been considered and investigated to circumvent the complexity of ocular anatomy and physiology.

The anterior part of the eye comprises the cornea, conjunctiva, anterior chamber, ciliary body, aqueous humour, lens and the lachrymal system, which forms a static and dynamic barrier to efficiently prevent foreign particles getting access into the eyes. As a result, non-invasive topical application, mainly through eye drops, leads to a low bioavailability with less than 3% of topically administered drug reaching the vitreous humour and even less in the posterior part of the eye (Boddu, Gupta, & Patel, 2014; Hughes, Olejnik, Chang-Lin, & Wilson, 2005). Several mAbs have been considered for local ocular application (Hu & Koevary, 2016; Ottiger, Thiel, Feige, Lichtlen, & Urech, 2009), with a clinical translation to humans that remains elusive and may require the use of penetration enhancers (see Section 3.2.1) (Thareja, Hughes, Alvarez-Lorenzo, Hakkarainen, & Ahmed, 2021). The posterior part is composed of the vitreous humour, retina, sclera and choroid. The cornea is the transparent front part of the eye, continuous with the sclera, which is composed of five layers comprising epithelium, stroma and endothelium. Since the cornea is negatively charged, it represents a primary barrier to topical administration using eye drops, especially for negatively charged drugs (Kim, Chiang, Wu, & Prausnitz, 2014). The other external parts of the eye, including conjunctival epithelium, sclera and choriocapillaries, represent absorption barriers for macromolecules coming from the systemic circulation with permeability dropping significantly for high-molecular weight drugs. In addition, the intra-ocular environment is protected from the outside by the blood-aqueous barrier and blood-retinal barrier. The blood-aqueous barrier is composed of the iris epithelium, iridial capillaries, ciliary muscle capillaries, and non-pigmented ciliary epithelium while the blood-retinal barrier located in the posterior part of the eye consists of retinal pigment epithelium and the endothelium of the retinal vessels. The two barriers comprise inter-cellular tight junctions restricting drug transfer between the eye and blood circulation (see Section 3.3) (Del Amo et al., 2017;

Thrimawithana, Young, Bunt, Green, & Alany, 2011), especially of proteins and other large molecules.

2.1.4.1. Delivery to the eyes: overcoming strategies. A major step forward in the treatment of retinal diseases was local delivery of antibodies, mainly targeting VEGF, in the internal parts of the eye, by intravitreal injection. It provides a high amount of antibody in the retina and vitreous compartments, while partly protecting the eyes and the rest of the body from antibody exposure. However, the volume of such injection should not exceed 0.1 or 0.2 mL in the anterior chamber or vitreal body, respectively, necessitating specific antibody formulation. Once injected, antibody diffusion is conditioned by the intrinsic characteristics of the vitreous humour, which is mainly composed by a hydrophilic matrix made of water, collagen and hyaluronic acid. The latter being negatively charged, it will restrict the diffusion of positively charged molecules (Q. Xu et al., 2013) (see Section 2.1.1). Bevacizumab (full-length IgG1) and ranibizumab (Fab fragment), two approved therapeutic mAb against age-related macular degeneration are both negatively charged under physiological conditions and will diffuse slowly through the vitreous cavity because of their molecular weight (Crowell et al., 2019). On the contrary, aflibercept (a fusion protein) which has a mild positive charge, will exhibit altered pharmacokinetic properties (Holash et al., 2002). The rheological properties of the vitreous humour are not homogeneous. Changes in the composition occur according to the position in the orbital cavity; the posterior part of the vitreous humour in contact with the retina has more collagen and hyaluronic acid that makes it less fluid-like. Age-related liquefaction of vitreous humour will also affect drug diffusion, especially for those of high molecular weight (Laude et al., 2010). Elimination of drugs after intravitreal injection might occur either by metabolism or by excretion through the systemic circulation. Anti-VEGF mAbs appear preserved from metabolism or degradation in the orbital cavity (Del Amo et al., 2017), but will be eliminated through diffusion to the aqueous chamber (Gal-Or et al., 2016) or the retinal pigment epithelium (Del Amo et al., 2017). Blood-ocular barrier does not allow permeation of antibodies from the systemic circulation, making the intravitreal injection a standard treatment achieving therapeutic drug levels in the retina. However, this invasive route of administration is associated with specific challenges including the crucial necessity to guarantee the absence of ocular toxicity. This requires the development of specific formulations (Elsaid, Jackson, Elsaid, Alqathama, & Somavarapu, 2016), which will allow prolonged action, eventually reducing injection regimen and improving tolerability.

2.1.5. ECM and intra-articular (IA) delivery of antibody

Joint inflammation is one of the leading causes for progressive motor disability. Osteoarthritis and rheumatoid arthritis are the most common forms of joint degeneration with high incidence requiring long-term therapeutic care (Lawrence et al., 1998). The main symptoms of these diseases are joint inflammation, pain and cartilage destruction. Standard care includes systemic administration of analgesic and anti-inflammatory agents, including anti-TNFα antibody (infliximab, etanercept, adalimumab) and anti-IL1β mAb (canakinumab). However, their chronic systemic administration regimen is frequently associated with substantial side effects (Tarner & Muller-Ladner, 2008). Synovial joints, most often affected by osteoarthritis and rheumatoid arthritis, are delimitated by a fibrous capsule and ligaments. They comprised bone endings covered by hyaline cartilage. The cartilage is an avascular tissue, composed of chondrocytes embedded in a highly anionic ECM, which is impermeable to molecules with a molecular weight >50 kDa, depending upon their charge and conformation (Foy & Blake, 2001). Because cartilage is avascular, it is inefficiently targeted by drugs administered systemically that must first reach the synovial fluid and then diffuse through the cartilaginous ECM. The synovial fluid mediates the supply of nutrients to the cartilage. Synovial fluid is mainly composed of hyaluronic acid and lubricin providing lubricating and frictionless mobility of the joint. To enter the joint from the systemic vasculature, a drug must pass through the capillary endothelium of the synovium, the ECM of the synovial intima and the macrophage-like synoviocytes composing the synovial membrane. Both cellular layers are highly fenestrated facilitating the directed exit of drug from the capillaries and the entry into the synovial fluid cavity. The consequence of this small diffusion barrier is a high synovial diffusion of molecules with a molecular weight <10 kDa. For larger molecules, fenestration will dictate a size-dependent restriction on the rate of passage that is 10 times less for IgG as compared to the one of albumin (Kushner & Somerville, 1971). During inflammation, capillary and synovial membrane permeabilities will increase, enhancing the entry of macromolecules in the synovial joint. This is exemplified by the increase in protein content in the synovial fluid from rheumatoid arthritis patients (Furst et al., 1999). The duration of residency in the joint of a therapeutic agent is dictated by the equilibrium between the rate at which the drug reaches and is cleared from the synovial fluid. On the contrary, the removal of macromolecules from joints, mainly driven by lymphatic drainage, is independent of size. Several studies tried to evaluate half-lives of various macromolecules including antibodies within the joints. Values reported ranged from 1–13 h for albumin, more than 25h for hyaluronic acid, 72h for gamma-globulin and 8–12 h for unspecified IgG (Scully et al., 2009). Joint inflammation will accelerate synovial clearance rate (Weinberger & Simkin, 1989). Indeed, for anti-inflammatory antibody, clinical PK data revealed half-lives of approximately 2–4 h (Evans, Kraus, & Setton, 2014).

2.1.5.1. Delivery to the joints: overcoming strategies. The IA administration is attracting as point-of-care therapy from both economics and patient's perspectives. It will achieve high drug concentration in the diseased area while limiting systemic exposure. Eventually, this will lead to a dose reduction and therefore minimization of costs and side effects. Despite these promising features, IA delivery for recombinant antibodies is rare; specific inhibition of the pro-inflammatory cytokines IL-1β and TNFα using anakinra, canakinumab or infliximab (both full-length and scFv fragment) delivered by IA have been clinically tested without conclusive results – which can be explained by the specific prevailing physiologic and anatomic conditions of the joint. The rapid clearance of antibody from the inflamed joint is a major limitation for therapeutics, requiring frequent injections that may be associated with unwanted side effects, patient discomfort and even morbidity. Specific formulations enabling controlled release over time are under consideration for injectable antibody in the joint. These include mainly natural polymers/lipids formulated as hydrogels as described for the anti-TNFα infliximab (Chen et al., 2020) and micro/nano particles (Eswaramoorthy et al., 2012) or *in situ* implants (Lambert & Peck, 1995), which have been used for other therapeutic proteins. However, such strategies do not come without disadvantage; indeed, controlled release formulations are associated with loss of bioactivity mainly due to stability issues (Koerselman et al., 2020). Finally, depending on their size, nanospheres might induce inflammatory reaction that may ultimately accelerate cartilage injury and even induce fibrotic processes (Kang & Im, 2014).

2.2. Proteases

Proteases are catalytic enzymes promoting peptide cleavage of proteins necessary for protein maturation, catabolism and the generation of amino acids. Their molecular pattern encompasses small enzymes (20–30 kDa) to sophisticated protein complexes, like proteasomes (0.7–6 MDa) (Lopez-Otin & Bond, 2008). Proteases are distributed in all living organisms including plants, animals and microorganisms such as bacteria and viruses. In humans, they constitute one of the largest families of enzymes with 2% of the genome encoding proteases. Proteases are classified into six different groups based on the specificity of their active site: aspartate-, cysteine-, glutamate-, metallo-, serine- and threonine-proteases. They can be found in all tissues with both

intra- and extra-cellular site of action. At physiological state, these enzymes are critically involved in the control of multiple biological processes including digestion, cellular communication and proliferation, haemostasis, immunity, wound healing and apoptosis. According to the irrevocability of their mode of action and the pleiotropic functions in which they are involved, proteases need to be tightly regulated. Several mechanisms control their production and secretion, including regulation of biosynthesis, activation of inactive precursors known as proenzymes or zymogens, and the binding of endogenous inhibitors and cofactors (Vasiljeva, Menendez, Nguyen, Craik, & Michael Kavanaugh, 2020). This allows the protease/anti-protease balance to be neutral, except in the gastro-intestinal tract where proteases participate to nutrients digestion, requiring sustained activation. The activation of resident immune cells and the recruitment of leukocytes generate tissue damage and extracellular matrix destruction resulting in a dysregulation of protease homeostasis. Imbalance in the regulation of proteases/proteases inhibitors plays a major role in the development of pathological conditions such as cancer, inflammatory, cardiovascular, neurodegenerative, bacterial, viral and parasitic diseases (Lopez-Otin & Bond, 2008). For example, in inflammatory bowel diseases, inflammation characterized in particular by a neutrophilic and macrophages infiltrate, leads to a highly proteolytic mucosal microenvironment. Neutrophil proteases (elastase and cathepsin G) and metalloproteinases (MMP3, MMP12, and MMP9) are highly up regulated in the inflamed gut (Baugh et al., 1999). In acute respiratory distress syndrome, significant elastase levels have been reported (Hashimoto et al., 2008). In chronic obstructive pulmonary disease (Pandey, De, & Mishra, 2017; Thulborn et al., 2019) and cystic fibrosis (Oriano et al., 2019; Witko-Sarsat et al., 1999), an increase in neutrophil-related proteases activity is observed and more particularly during infectious exacerbations contributing to the decline of respiratory function (Chalmers et al., 2017). In cancer, protracted activation of extracellular proteases, with MMPs representing the most prominent family associated with tumorigenesis, provides additional survival advantages to cancer cells (Kessenbrock, Plaks, & Werb, 2010). Proteases promote angiogenesis, impair anticancer lymphocytes functions and facilitate metastasis through tissue remodelling.

Proteases may be considered as a biological barrier complicating the administration of therapeutic proteins especially in disease conditions. Extracellular proteases such as neutrophil proteases, MMPs or bacteria secreted proteases (Dunnhaupt, Kammona, Waldner, Kiparissides, & Bernkop-Schnurch) have the ability to cleave endogenous immunoglobulins (Brezski & Jordan, 2010; von Pawel-Rammingen, Johansson, & Bjorck, 2002). Several studies have demonstrated such proteolysis both *in vitro* and *in vivo*, hypothesizing that bacterial-secreted or tumour-associated proteases may act as virulence factors to escape the immune response (Plaut, Wistar Jr., & Capra, 1974; Ryan et al., 2008; Senior & Woof, 2005). The proteolytic inactivation of therapeutic antibodies is under-appreciated as a contributor to therapeutic response evasion, but several studies highlighted the critical role of proteases on mAb PD. Proteases (MMP-3, MMP-12 and HNE) affect the integrity of anti-TNFα mAb and compromise their therapeutic capacities in comparison with intact mAb (Biancheri et al., 2015; Curciarello et al., 2020). Proteolysis of mAbs (IgG1 and 4) by MMPs (MMP-12) and IdeS has been shown to be dependent on the incubation time (Deveuve, Lajoie, Barrault, & Thibault, 2020), with long time exposition to proteases being responsible for a decreased efficacy of the anti-HER2 antibody pertuzumab (Hsiao, Fan, Jordan, Zhang, & An, 2018; Kinder et al., 2013). A single proteolytic cleavage in the lower hinge of a humanized IgG1, trastuzumab, or pertuzumab, by MMP-12 also led to a loss of one of the Fc fractions, reducing its effector activity via the immune system and therefore its anti-tumour efficacy *in vivo* (Fan et al., 2012; Hsiao et al., 2018; Zhang et al., 2015).

2.2.1. Proteases: overcoming strategies

The identification of proteolytic cleavage sites could allow the development of mutant antibodies protected against proteases. Indeed, the fact that IgG2 are resistant to MMPs contrary to IgG1 has been associated with differences in amino-acid sequence of the lower hinge region. Specific mutations in the CH2 domain result in resistance to MMPs with restored *in vitro* complement-dependent cytotoxicity and antibody-dependent cellular phagocytosis activities. Thus, engineered antibodies that are resistant to hinge proteolysis hold promise to maintain potent effector functions (Kinder et al., 2013). Another strategy would rely on the use of rescuing antibodies, which bind to hinge neo-epitope generated after proteolytic cleavage and restore the effector functions of the protease-sensitive antibody. This was elegantly demonstrated by Fan et al. with an antibody targeting the hinge neo-epitope of trastuzumab generated by MMP or IdeS proteolysis. Fan et al. demonstrated both *in vitro* and *in vivo* that the rescuing antibody restored antibody-dependent cellular cytotoxicity function of trastuzumab as well as the recruitment of immune effector cells into the tumour microenvironment (Fan et al., 2015). *In vitro*, co-incubation with specific protease inhibitors inhibits antibody proteolysis (Curciarello et al., 2020). *Ex vivo*, TNFα-neutralizing function was restored after incubation of anti-TNFα mAb (infliximab and adalimumab) with MMP inhibitors in the sera from patients with inflammatory bowel disease (Biancheri et al., 2015). These co-treatment strategies with protease inhibitors may represent an interesting way to maintain the antibody functionality in inflammatory contexts. However, numerous proteases coexist in tumour/inflammatory/infected microenvironments complicating the choice of inhibitors as well as their optimal concentration that would need to be defined and adjusted to each pathological context.

2.3. Other extracellular barriers

2.3.1. Mucus and oral or airway administration of antibody

Mucus is a viscoelastic hydrogel located in the airways, the eyes, the gastrointestinal, urogenital and reproductive tracts, and the peritoneal surface of intra-abdominal organs. Continuously produced and secreted by mucous cells, it coats the luminal surface of these different compartments acting as a lubricant and maintains a hydrated layer above the epithelial cells (Leal, Smyth, & Ghosh, 2017). On exposed surfaces, mucus also provides a barrier to pathogens and foreign substances including environmental particles (Lai, Wang, Wirtz, & Hanes, 2009). Mucus is mainly composed of water (>95%), mucins, proteins, DNA, cells and cellular debris (Leal et al., 2017). Mucins are the primary non-aqueous component of mucus. They give to the mucus its three-dimensional mesh network structure mainly stabilized by covalent and noncovalent interactions, including hydrophobic, electrostatic, hydrogen bonds, or other specific binding interactions (Creeth, 1978; Lieleg & Ribbeck, 2011). The homeostasis of its components is highly interdependent and govern mucus main physicochemical properties, as pore size, viscoelasticity, pH, ionic strength and charge (Leal et al., 2017). For example, viscosity may be modified by the hydration, pH level and salt, proteins and ionic concentration (Frohlich & Roblegg, 2014).

It is obvious to imagine mucus acting as a barrier to the transport of drugs and therapeutic molecules administered through oral or inhaled route (Bansil & Turner, 2006; Leal et al., 2017). However, several studies described endogenous immunoglobulins behaviour in respiratory, GI and cervico-vaginal homeostatic mucus suggesting that their diffusion was barely altered in mucus (Olmsted et al., 2001). Intriguingly, the mucus-antibody interplay at mucosal surfaces also acts as a new defence mechanism of antibody during infections. Indeed, pathogen-coated antibodies form weak and transient Fc-mucins bonds – via sugars– and trap pathogens, thereby preventing pathogens to infect epithelial cells and facilitating their physical elimination through mucociliary clearance (Schaefer & Lai, 2021; Wang et al., 2017; Yang et al., 2018). The “muco-trapping function” of antibodies-coated to pathogens has been nicely exemplified with virus-like particles (VLP) of influenza-binding IgG, and the anti-Ebola antibody cocktail ZMapp bound to Ebola VLP in fresh airway mucus from healthy adult. Overall,

"muco-trapping" function of antibody appears as a universal effector function for IgG in major mucus and apply to other immunoglobulin subclasses and extracellular matrix. However, muco-trapping property of antibody implies on a subtle balance partly depending on the rapid IgG diffusion within mucus and binding to pathogen. To date, the capacity of antibody Fc-mucin to crosslink and immobilize pathogen has never been studied in pathological conditions, when mucus is altered. Indeed, in broncho-obstructive diseases such as cystic fibrosis and chronic obstructive pulmonary disease, overproduction of mucus is associated with dehydration and a shrinkage of pore size (Duncan et al., 2016). In cancers, mucins overproduction and heterogeneity has been associated to tumour progression (Hukill & Vidone, 1965). This resulted in modifications of architectural structure and viscoelasticity of the mucus present in the cancer microenvironment and has been described in the context of colonic and gastric adenocarcinoma (Hollingsworth & Swanson, 2004). Inflammatory disorders such as inflammatory bowel disease may also impact mucus architecture secondary to a modification of mucins' glycosylation changing viscoelastic properties, resistance to bacterial degradation, and adhesion (Corfield, Carroll, Myerscough, & Probert, 2001). In summary, mucus structure is often abnormal in various pathological conditions and may impair antibody diffusivity, as previously described for other therapeutics (Braeckmans, Peeters, Sanders, De Smedt, & Demeester, 2003; Ensign et al., 2012; Maisel et al., 2016).

2.3.1.1. Mucus: overcoming strategies. The development of antibodies able to diffuse through the mucus requires an exhaustive knowledge of its structure and its physicochemical properties both in physiological and pathological contexts. Depending on the location of the antibody target, it may be necessary to explore the impact of formulations or co-administrated treatments to improve their deposition and define their efficacy after administration in the nasal, lung and oral mucosa. When the target is located into the mucus or in the organ lumen, formulations conferring mucoadhesive properties on the treatment and prolonging its local stability may be preferred. For example, encapsulation with polymeric non-charged and lipophilic nanoparticles optimizes mucoadhesion, cellular uptake, immune system interactions and cell targeting (Ensign, Cone, & Hanes, 2012). Conversely, when the target is located under the layer of mucus, strategies improving mucosal permeability of drugs must be favoured. These strategies may include the use of mucolytic treatments such as recombinant human deoxyribonuclease (rhDNase) (Macierzanka et al., 2014; Sanders et al., 2000) or N-Acetyl cysteine (Suk et al., 2011; Vukosavljevic et al., 2017) as co-treatments. Nanoparticle-based systems may also be of particular interest allowing protection of drug from degradation mechanisms and the control of surface properties to avoid adhesion or steric hindrance (Dunnhaupt, Kammona, Waldner, Kiparissides, & Bernkop-Schnurch, 2015; Ensign, Cone, & Hanes, 2012; Lai, Wang, & Hanes, 2009). Poly(lactic-co-glycolic acid) PLGA or poly(ethylene glycol) PEGylation have shown their effectiveness to enhance mucus transport of drugs in gastric, intestinal, vaginal, cervicovaginal, respiratory animal mucus and in CF sputum (Suk et al., 2009; Tang et al., 2009; Wang et al., 2008; Yu et al., 2012). Chitosan, a cationic polymer, is also used for oral and nasal drug applications in particular to improve the administration of nasal vaccines (Csaba, Garcia-Fuentes, & Alonso, 2009). Presently, we do not know if it would be relevant to investigate such strategies, since the impact of the pathological context on antibody diffusivity and muco-trapping function of mAb in the mucus remains unknown.

2.3.2. pH and oral administration of antibody

The gastrointestinal tract is essential in digestion and absorption of nutrients while, as other mucosae, it must defend the host against exogenous materials and microorganisms. Consequently, it harbours several non-cellular barriers, which renders oral administration of antibody challenging. After oral administration, drugs are conducted from the mouth to stomach, small intestine and finally conveyed to the colon and expelled into faeces. Each gastrointestinal segments exhibit specific environmental conditions, which may alter antibody efficacy. In the stomach, antibody is subjected to degradation by pepsin, which is activated by the acidic pH (pH=2). The acidic pH will also promote irreversible changes in protein conformation (Lopez et al., 2019). In the small intestine, antibody will be exposed to proteases activated by pH ranging between 6.0–7.3 like trypsin, chymotrypsin, carboxypeptidase, and elastase (see Section 2.2).

2.3.2.1. Oral delivery: overcoming strategies. Therapeutic proteins and peptides are poorly resistant to the acidic and proteolytic content of the stomach, thus protection strategies have been envisioned to overcome these barriers and include enteric coating (Crowe et al., 2019) and encapsulation in nanoparticles (Tashima, 2021). Interestingly, formulations based on coated mini capsules were engineered for the local treatment of inflammatory bowel disease with anti-TNFα antibody and led to high intestinal and colon concentrations with limited systemic exposure (Crowe et al., 2019). Thus, mini capsules may have a huge potential to improve the management of inflammatory bowel disease, which currently require chronic parenteral administration associated with deleterious systemic side effects. Omeprazole, a well-known proton pump inhibitor neutralizing stomach acidity, has been proposed in combination with anti-CD3 mAb foralumab and muromomab, in the treatment of type 2 diabetes and ulcerative colitis, respectively (Boden et al., 2019). Larger studies are required to confirm the benefits of the combination.

2.3.3. Biofilm and anti-infectious antibody

A key virulence factor associated with the development of chronic infections is the ability of pathogens to build organized cells communities (Costerton et al., 2003; Hoiby, Ciofu, & Bjarnsholt, 2010). Biofilms are three-dimensional structured communities of pathogens encased in a self-produced extracellular matrix (Neu et al., 2010), which can adhere on both inert and biotic surfaces (Bowen, 2016; Veerachamy, Yarlagadda, Manivasagam, & Yarlagadda, 2014). Biofilms are mainly composed of an extracellular polymeric substance (del Aguila, Longstreth Jr., McGuire, Koepsell, & van Belle, 2003) including exopolysaccharide (Ciornei et al., 2010), extracellular proteins (Fong, Karplus, Schoolnik, & Yildiz, 2006; Tielker et al., 2005) and extracellular DNA (eDNA) (Montanaro et al., 2011). The importance of the structural biofilm complexity as pathological obstacle to antimicrobial therapeutics is now widely recognized. This recalcitrance is a direct consequence of the physical and biological properties of biofilm. Exopolysaccharides represent 90% of biofilm's biomass, which composition is continuously evolving and differs depending on the bacteria species, and form a diffusion-limiting barrier impairing the penetrance of therapeutics and binding to their respective targets. Thanks to an electrostatically charged net, biofilm is also able to either repulse or trap antimicrobial peptides and proteins (Anaya-Lopez, Lopez-Meza, & Ochoa-Zarzosa, 2013) contributing to its antimicrobial resilience (Koo, Allan, Howlin, Stoodley, & Hall-Stoodley, 2017). Furthermore, a large array of molecules released near the biofilm enable chemical inactivation or degradation of anti-microbial agents (Hall & Mah, 2017; Lewenza, 2013; Stewart & Franklin, 2008). Biofilm also reduces the efficiency of the immune response, especially phagocytosis mediated by macrophages or neutrophils. Target-mediated recognition of microbial molecular pattern by Ig activates phagocytes, which have been recruited to the site of infection, in a phenomenon referred as opsonophagocytosis. However, the steric hindrance induced by exopolysaccharide limits the engulfment of pathogens by phagocytes, thus impeding microbial clearance (Campoccia, Mirzaei, Montanaro, & Arciola, 2019). Therefore, bacteria embedded in biofilm are protected against endogenous antibody-mediated opsonophagocytosis. Finally, in addition to its role as a barrier for penetration, reduced efficacy of antimicrobial treatment against biofilm is also due to the status of cells present inside the biofilm: a large proportion is in a status of reduced metabolism (Wood,

Knabel, & Kwan, 2013) whereas most anti-microbial agents target active cellular processes, including cell wall formation, virulence factors expression, translation or transcription (Mah & O'Toole, 2001). Altogether, these processes, associated with an important heterogeneity of phenotype and genotype of cells embedded in biofilms (Becker, Hufnagle, Peters, & Herrmann, 2001; Besharova, Suchanek, Hartmann, Drescher, & Sourjik, 2016; Cho & Caparon, 2005; Stewart & Franklin, 2008), promote the development of resilient pathogenic populations (Crabbe, Jensen, Bjarnsholt, & Coenye, 2019). Up to now, no demonstration has been provided on the impact of biofilm on antibody ability to reach their pathogenic target. It would be important to take into account the biofilm barrier in the development of anti-infectious antibody.

2.3.4. Tumour blood flow and interstitial pressure

Role of branching and expansion of blood vessels in tumour growth do not need to be proved any longer. However, rapid growth of tumour cells is often unsynchronised from effective vasculature growth, which results in non-vascularised tumour areas (Durand & Aquino-Parsons, 2001). This lack of tumour blood flow near new tumour areas can hinder drug delivery and in particular therapeutic antibodies as the majority of immunotherapies are administered systemically. Thurber and Weissleder in 2011 (Thurber & Weissleder, 2011) have shown that antibody uptake depends on both tumour vasculature and blood flow using sub-saturating antibody concentrations, and on the number of receptors using saturating antibody concentrations. Decrease in vessel diameters, as branching progresses, induces a decrease of blood pressure and blood flow. Moreover, unorganised vessel structure in the tumour microenvironment is associated with blood flow resistance due to vessels tortuosity (irregular network structure), loose of fluidity for red blood cell (hypoxia, acidosis) and retrograde or intermittent blood flow due to anarchic vessel growth. Drug transport by the systemic route is, as a result, considerably impeded (Jain, 1988). A normalization of the blood flow velocity may help to recover a "normal" diffusion. Gao and his team (Gao et al., 2020) have shown that an increase of blood flow velocity enhanced pressure gradient between vessels and interstitial tissue, thereby promoting diffusion of nanoparticles from the blood to the interstitial domain. On the opposite, interstitial fluid pressure in tumour microenvironment is higher than in normal tissue (Gao et al., 2020; Wu et al., 2014). Indeed, an easy transfer of macromolecules from the blood to the interstitial space due to fewer tight junctions and lack of pericyte coverage lead to high pressure (Kerbel, 2006). In addition, a lack of lymphatic function in tumour system reduces the expulsion of fluid and result in high pressure (Padera et al., 2002). In addition to the defective blood and lymph vessels, the increased contractility of cancer-associated fibroblasts and a dense ECM compartment both contribute to the increase and persistence of high interstitial fluid pressure in tumours (Eikenes, Tari, Tufto, Bruland, & de Lange Davies, 2005; Heldin, Rubin, Pietras, & Ostman, 2004). In the context of pancreatic adenocarcinoma, high hyaluronic acid content in the microenvironment has been correlated to increased water molecules retention in the stroma and increased interstitial fluid pressure (Jacobetz et al., 2013). Interstitial fluid pressure further inhibits the transport of drugs from the blood vessels (Ferretti, Allegrini, Becquet, & McSheehy, 2009). Heine et al. (2012) reported a heterogeneous distribution of an anti-EpCAM MOC31 mAb in subcutaneous xenografts with only few regions in the vicinity of perfused blood vessels positive for the mAb. The reduced penetration was correlated to the observation of immature blood vessels together with sparsely existing lymph vessels and an elevated interstitial fluid pressure (three time higher as compared to normal tissue).

2.3.4.1. Blood flow and interstitial pressure: overcoming strategies. Strategies to lower tumour interstitial fluid pressure and regulate blood flow should be considered in order to improve antibody distribution and therapeutic effectiveness. Actually, antiangiogenic mAbs (anti-VEGF or anti-VEGFR2) were used to restore interstitial fluid pressure and normalize anarchic blood vessels growth in order to enhanced small-drug penetration in tumour (Jain, Tong, & Munn, 2007; Tong et al., 2004). The relevance of this strategy in the improvement of anti-cancerous mAb remains to be clinically evaluated.

3. FcRn and cellular barriers

3.1. The role of FcRn

Recombinant antibodies need to cross different cellular barriers to reach the organs in which their target is expressed. All these barriers have in common the expression of the neonatal Fc receptor (FcRn) described by Brambell in 1964 (Brambell, Hemmings, & Morris, 1964) and known since then as a major player in IgG half-life and biodistribution. By analogy to IgG, mAb (and Fc-conjugated protein) PK/PD is also submitted to FcRn control. FcRn is composed of a heavy chain in non-covalent association with β2-microglobulin. The heavy chain is made up of 3 extracellular domains (α1, α 2 and α 3), a transmembrane domain and an intracellular tail. FcRn is an intracellular receptor (over 90%) expressed in the cellular early endosomes (Antohe, Radulescu, Gafencu, Ghetie, & Simionescu, 2001; Ober, Martinez, Vaccaro, Zhou, & Ward, 2004). It is transiently expressed on the cell surface during endosome fusion to the membrane. Indeed, only about 4% of the FcRn pool is expressed at the surface with 2% FcRn non-internalisable by cells (D'Hooghe, Chalmers, Heywood, & Whitley, 2017). FcRn allows the recycling of its two ligands, IgG and albumin, in order to prolong their half-life in blood and tissues. Following their entry into the cell by pinocytosis, monomeric IgG (comprising mAb) and albumin bind to FcRn in a pH-dependent manner, at acidic pH (6.0–6.5) in early endosomes. This pH-dependent binding is mediated by the interaction of the FcRn with residues located in the CH2–CH3 hinge region of the IgG (Kim et al., 1999; Oganesyan et al., 2014; Raghavan, Bonagura, Morrison, & Bjorkman, 1995). IgG are then released at neutral pH into the circulation (Ober et al., 2004). This phenomenon confers protection of IgG from lysosomal degradation and prolongs their half-life (Ko, Jo, & Jung, 2021). The same mechanism is also involved in FcRn-dependent IgG transcytosis in polarized cells or across various tissues (Antohe et al., 2001; Dickinson et al., 1999; Kim et al., 1999).

Interestingly, polymorphism of the FcRn gene may influence the PK/PD of IgG, including therapeutic mAb. FcRn polymorphism, consisting in variable number of tandem repeats (VNTR, five different alleles VNTR1–VNTR5) within the *FCGRT* promoter, influences the quantity of FcRn transcripts (Sachs et al., 2006). Indeed, VNTR3/3 homozygous individuals express more FcRn transcript than VNTR2/3 heterozygous individuals. Finally, VNTR3/3 polymorphism leads to better management of IgG in monocytes due to an increase binding of IgG (Sachs et al., 2006). In this context, Passot et al. showed that VNTR polymorphism was associated with variations in cetuximab PK parameters. Indeed, VNTR3 homozygote patients (high level of FcRn transcripts) had a lower cetuximab distribution clearance than VNTR2/VNTR3 (low level of FcRn transcript) and VNTR3/VNTR4 patients (Passot et al., 2013). VNTR in FcRn promoter also influences the PK of intravenous Ig (IVIg) in patients with Guillain-Barré Syndrome (GBS) or IVIg efficiency in Common-Variable-Immunodeficiency (CVID) patients (Fokkink et al., 2016; Gouilleux-Gruart, et al., 2013).

FcRn is a receptor found in a large variety of epithelial cell types such as hepatocytes, enterocytes, thyrocytes and podocytes. FcRn is also expressed in endothelial and hematopoietic cells (Akilesh, Christianson, Roopenian, & Shaw, 2007; Borvak et al., 1998; Haymann et al., 2000; Israel et al., 1997; Latvala, Jacobsen, Otteneder, Herrmann, & Kronenberg, 2017; Montoyo et al., 2009). This wide cellular expression leads to its ubiquitous presence in organs all over the body, consistent with the functions attributed to FcRn regarding therapeutic antibodies and with its role in overcoming cellular barriers. Its function in each specific tissue will be discussed in the sessions below.

3.2. Epithelial barrier and mucosal delivery of antibody

Non-invasive routes have been investigated for therapeutic antibodies using delivery through mucosa including nasal (Rohrer, Lupo, & Bernkop-Schnurch, 2018), lung (Respaud, Vecellio, Diot, & Heuze-Vourc'h, 2015), buccal (Montenegro-Nicolini & Morales, 2017) and oral mucosa (Vllasaliu, Thanou, Stolnik, & Fowler, 2018) for either topic or systemic-acting drugs. Mucosal surfaces represent an extensive area that face the external environment, require the establishment of anatomic and physiologic barriers, and prevent pathogens and macromolecules from reaching the internal surfaces of the body.

The epithelium represents a barrier for both locally and systemically acting antibody, depending on the antigen target location and route of administration (Ferrati, Wu, Kanapuram, & Smyth, 2018; Homayun, Lin, & Choi, 2019). Mucosal epithelium, which is almost a non-keratinized epithelium (except in specific areas of the oral cavity), is a critical barrier that provides protection for pathogens entering the body. The regulation and exchange of molecules between the environment and the underlying tissues by epithelium is exemplified by nutrient absorption in the gastro-intestinal tract (Turner, 2009) and gas exchange in the airways (Hellings & Steelant, 2020). Directional transport across the epithelium is provided by cellular polarity featuring apical and basolateral domains that are asymmetrical in structure and functions (Farquhar & Palade, 1963). From the apical to basolateral side, the apical junctional complex is composed of tight junctions, adherent junctions, gap junctions and desmosomes (Fig. 2). It is the main regulator of molecular transport across the epithelium. Indeed, epithelium tight junctions dictate the characteristics of paracellular permeability that accepts, with high capacity, molecules with molecular mass <600 Da and size in the range of 8–9 Å in diameter (Watson, Rowland, & Warhurst, 2001). During transient alterations of the epithelium, a leak pathway has also been characterized, allowing less restrictive, low capacity and size-independent transportation (Buschmann et al., 2013; Turner, 2009). Due to their large molecular size and high polarity, paracellular transportation of full-length (Guilleminault et al., 2014) and fragments antibodies (Patil et al., 2018) is estimated low or negligible. Besides paracellular transportation, transcellular transport of larger molecules can occur at the mucosal surface. Macromolecules localised in the lumen can access the lamina propria by specialised cells including microfold cells (in the gastrointestinal tract) and dendritic cells (Kimura, 2018) or by unspecific endocytosis through epithelial cells. Endocytosis may result in partial or total degradation of proteins in acidic and lysosomal compartments (Heyman, Ducroc, Desjeux, & Morgat, 1982). Transcellular transport of antibody (with an Fc domain) mainly occurs through receptor-mediated mechanisms involving FcRn (see section 3.1). In lung mucosa, FcRn is expressed in bronchial and alveolar epithelial cells (Akilesh et al., 2007; Latvala et al., 2017; Sakagami et al., 2006; Spiekermann et al., 2002) where it maintains homeostatic IgG levels (Vogelzang et al., 2016) and allows IgG transcytosis across the mucosal surface (Spiekermann et al., 2002). For inhaled antibodies, FcRn contributes to mAb biodistribution via recycling and transcytosis in the airways (Guilleminault et al., 2014). FcRn is also expressed in human nasal epithelium (Campanari, Bourefis, & Kabashi, 2019) and may mediate transcytosis of mAb across this tissue after nasal administration (Bequignon et al., 2019).

3.2.1. Epithelial barrier: overcoming strategies

According to the target localisation, several strategies have been designed to interface with mucosal surfaces in an attempt to improve or reduce penetration of antibody through the epithelial barrier. Structural simplification of antibody architecture has proven to improve *in vivo* delivery after inhalation. Indeed, anti-IL-13, anti-TNFR1 or anti-RSV F protein antibody fragments display improved tissue penetration and biodistribution after inhalation (Bertok et al., 2012; Detalle et al., 2016; Hacha et al., 2012). Several studies describe the use of delivery carrier systems to overcome the epithelial barrier for proteinaceous drugs. This includes permeation enhancers that will temporarily weaken tight junctions enhancing paracellular permeability (Maher, Mrsny, & Brayden, 2016). They have been successfully used for insulin, liposomes and nanoparticles, but only one study reported a positive impact on IgG delivery. The concomitant intranasal administration of (^{125}I) I-IgG and MMP-9 significantly improved the uptake of antibody into the brain as compared to unformulated antibody (Kumar et al., 2018). However, permeability enhancers should be used with caution as mucosal irritation and toxicity has been reported (Maher et al., 2016; Marttin et al., 1997). Another method consists in improving transcellular transport using cell-penetrating peptides: short-length (5–30 amino-acids) peptides, rich in positive charges and containing hydrophobic tryptophan residues may help translocation across epithelium. This technology has been successfully used mainly for nanotechnologies (Jin et al., 2012; Porsio, Craparo, Mauro, Giammona, & Cavallaro, 2018) and is currently under investigation for antibody delivery across the mucosal epithelium (Zhang et al., 2018; Zhao, Brown, Kohler, & Muller, 2003). When aiming luminal target, prolonging antibody retention may be of particular interest. Conjugation of polyethylene glycol (PEG) chains to antibody will increase the molecular weight lowering transcytosis to the systemic circulation (Patil et al., 2018) by inhibiting its binding to FcRn, as demonstrated for the certolizumab pegol (Mariette et al., 2018). In addition, it will enhance stability and mucoadhesion, while avoiding the uptake by phagocytes (see Section 2.3.1.1) improving antibody PK profile (Koussoroplis et al., 2014). When considering the mucosal route of administration to deliver a drug acting at the systemic level, it would be beneficial to use the FcRn-mediated transcytosis. Indeed Fc-conjugated drugs, like Epo-, IFNb- of FSH-Fc have shown both improved transepithelial transport or half-life in the airways and in the plasma (Rath et al., 2015). However, it is noteworthy that antibody epithelium-to-endothelium diffusion after inhalation delivery is slow, indicating that other parameters may contribute to airway-blood passage (Guilleminault et al., 2014; Respaud et al., 2016).

3.3. Endothelial barrier and systemic delivery of antibody

The endothelium is a cell monolayer lining blood vessel walls that not only controls the vascular tone, haemostasis and angiogenesis but also cell behaviour, innate immunity, cell-cell interactions, and cell metabolism in the vessel wall (Xu et al., 2021). Vascular endothelium cells act like a gatekeeper controlling the flow of molecules and fluid into and out of a tissue. In this section, we will discuss how vascular endothelium and more specifically the blood-brain barrier (BBB) limit antibody delivery.

3.3.1. Structure of blood vessels

The structure of blood vessels is commonly described with three tunicae characterised by morphological differences: intima, media and adventitia (from the most internal to the most external tunic). Microvasculature, the main place for exchanges, is composed by intima tunica with endothelial cells for the most important part and pericytes localized at the outside with basal membrane. The growth of this network is dependent of angiogenesis phenomenon, which allows neo-vessels growth from pre-existing structures (one of the ways used by tumour cells to increase neo-vessels proliferation) (Raza, Franklin, & Dudek, 2010; Tennant & McGeachie, 1990). Blood vessels are essential to protect tissues from molecules present in the bloodstream circulation. Nevertheless, a non-negligible proportion of drugs must diffuse through the micro-vessel wall to reach their targets. Drug diffusion depends both on micro-vessel structure and on drug diffusion abilities. Due to their features (hydrophilicity and large size), antibodies are restricted in their ability to pass through endothelial vessel cells. Studies in mice have shown that FcRn is expressed in the endothelium of small arterioles and capillaries, but not in larger vessels like central vein and portal vasculature (Borvak et al., 1998). FcRn is expressed on the walls of endothelial cell endocytosis vesicles (pinocytosis) in the human vascular

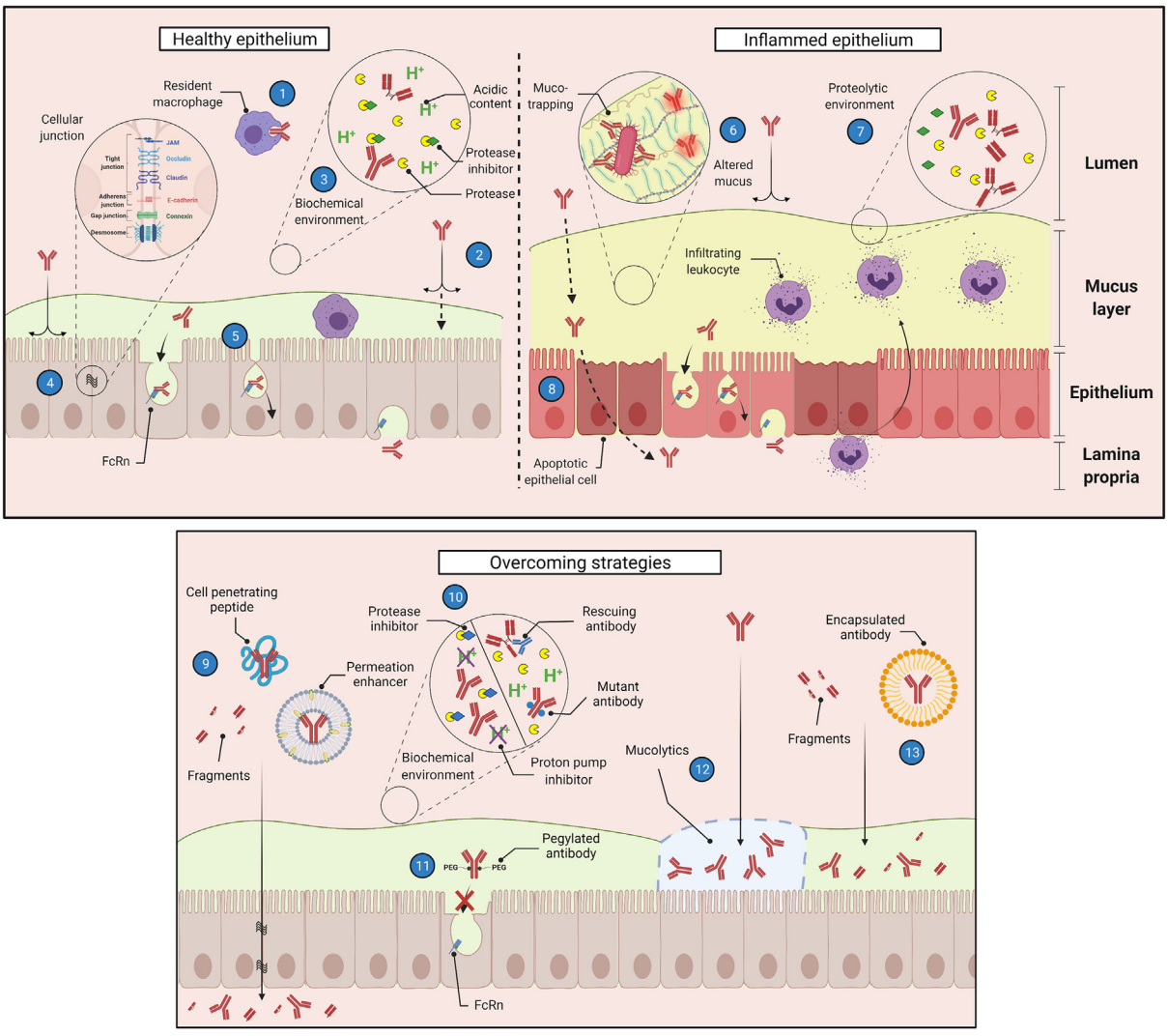

Fig. 2. Schematic representation of epithelial barriers to antibodies in healthy and disease conditions.
At steady state (left panel), mAbs present in the lumen may encounter biological barriers at the mucosal surface limiting their bioavailability. Resident macrophages (1) patrolling the mucosa could phagocytose mAbs leading to their degradation or denaturation. The mucus (2) covering the epithelium can partly repel Abs. In homeostatic conditions, balance between proteases and anti-proteases is neutral (3), except in the gastro-intestinal tract where the acidic pH will activate specific proteases and alter mAb conformation. Once the epithelium is reached, tight junctions between epithelial cells (4) prevent paracellular transport of mAb. mAbs can cross the epithelial surface by transcytosis, mainly through their binding with FcRn (5) resulting in improvement or decrease in the mAb bioavailability depending on the location of its target. In inflammatory conditions (right panel), mucus structure and composition are altered (6) with thicker layer and tighter pores decreasing the diffusion of mAbs. The activation of resident immune cells and the recruitment of leukocytes generate a dysregulation of proteases / protease-inhibitors balance (7) leading to a proteolytic environment favoring mAb proteolysis. Local inflammation leads to cellular damage and epithelial apoptosis promoting para- and trans-cellular leakage of mAbs (8). Several strategies have been investigated trying to solve mucosal barrier problems. In order to circumvent epithelium impermeability, cell-penetrating or permeation enhancer strategies have been considered as well as reducing mAb size, using small fragments, in order to improve mAb trans-epithelial passage (9). The use of protease inhibitors, proton-pump inhibitors or rescuing mAb may protect from degradation and denaturation (10). When considering local target, mAb-pegylation will lower transcytosis and increase mAb retention within mucosa (11). In order to improve mAb diffusion through the mucus, the addition of mucolytics (12), the encapsulation of mAb in nanovectors or the use of small fragments have been investigated (13).

endothelium (Paintaud, 2009). After membrane invagination, mAb bind this specific receptor. FcRn expression in blood vessels leads to sustainable IgG serum half-life by recycling them to the blood, including mAb (Pyzik et al., 2019). For tumour vasculature, there is a paradoxical situation. On the one hand, the inflamed environment combined with the rapid tumour vasculature growth induce an increase in the number and size of gaps, a decrease in the quality of basal membrane and a reduced number of pericytes that facilitates diffusion through intima tunica of tumour microenvironment capillaries (Azzi & Gavard, 2014; Ribatti et al., 2005). On the other hand, tumour vasculature tortuosity and unorganised microcirculation structure decreases diffusion capacity by flow resistance mechanism. In fact, even if the vascular density is high in tumour microenvironment, thanks to hypersecretion of growth factor (VEGF for example), the unstructured network could be an important obstacle for drugs delivery (Dewhirst & Secomb, 2017), as illustrated in Section 2.c.iv for antibodies.

3.3.2. The blood-brain barrier (BBB): the wall between the blood and the central nervous system (CNS)

The CNS is protected by three main barriers that separate it from the rest of the body: the BBB, the blood-cerebrospinal fluid barrier, and the arachnoid barrier. The BBB is found at the interface between the cerebral systemic circulation and the brain (Wolburg, Noell, Mack, Wolburg-Buchholz, & Fallier-Becker, 2009; Zlokovic, 2008). Given the high sensitivity of neurons to changes in their microenvironment and the low regenerative capacity of the nervous system, a disruption of the BBB can have detrimental effects on the CNS. The BBB strictly modulates the passage of molecules into and from the brain parenchyma.

This barrier is mainly made up of specialised endothelial cells characterised by the lack of fenestrae, reduced pinocytosis and transcytosis, the expression of specific metabolising enzymes, and the presence of energy-dependent efflux transporters, such as the FcRn, ATP-binding cassette transporters and low-density lipoprotein receptor-related protein 1 (LRP1) (Deane et al., 2004; Higgins, 2001; Salameh & Banks, 2014). Therefore, these cells demonstrate a high transendothelial electrical resistance, coupled with low paracellular and transcellular permeability (Maherally et al., 2018). This electrical resistance is largely due to the presence of continuous junctional complexes, and particularly tight junctions between endothelial cells (Bazzoni & Dejana, 2004). Tight junctions enhance the selective transport of molecules across the BBB through two main mechanisms: (i) conferring endothelial cells their polarity by separating their apical and basolateral surfaces, each of which has a different expression of transporters, and (ii) restricting paracellular transport pathways and forcing molecules to move transcellularly through specific transporters (Tsukita, Furuse, & Itoh, 2001). The BBB endothelial cells are surrounded by pericytes and perivascular astrocytic end-feet. Pericytes share the basal lamina of endothelial cells and act like vascular smooth muscle cells to regulate blood flow through BBB capillaries (Jespersen & Ostergaard, 2012). They also release signalling molecules that modulate tight junctions' density and astrocytic end-feet polarisation (Armulik et al., 2010) (Fig. 3). The basal lamina is in turn composed of a mixture of laminin, fibronectin, type IV collagen, and heparin sulphate, which further regulate the permeability of the BBB (see Section 2.1.1) (Milner et al., 2008). The BBB is not static and is finely modulated in both healthy and disease conditions. Its permeability is influenced by several cell types including endothelial cells, pericytes, astrocytes, microglia, and neurons, which morphologically and molecularly interact together to form the functional neurovascular unit (Luissint, Artus, Glacial, Ganeshamoorthy, & Couraud, 2012).

It is estimated that less than 0.1% of systematically administered antibody enter the CNS through nonspecific pathways (Atwal et al., 2011; Poduslo, Curran, & Berg, 1994). An even lower amount of 0.009% of the injected dose reaches the cortex (St-Amour et al., 2013). This may be partly attributable to the presence of FcRn (Cooper et al., 2013). In the BBB, FcRn is expressed in microvascular endothelium and in choroid plexus epithelium that constitute the BBB (Schlachetzki, Zhu, & Pardridge, 2002), but not in neurons, astrocytes, or microglia (Akilesh

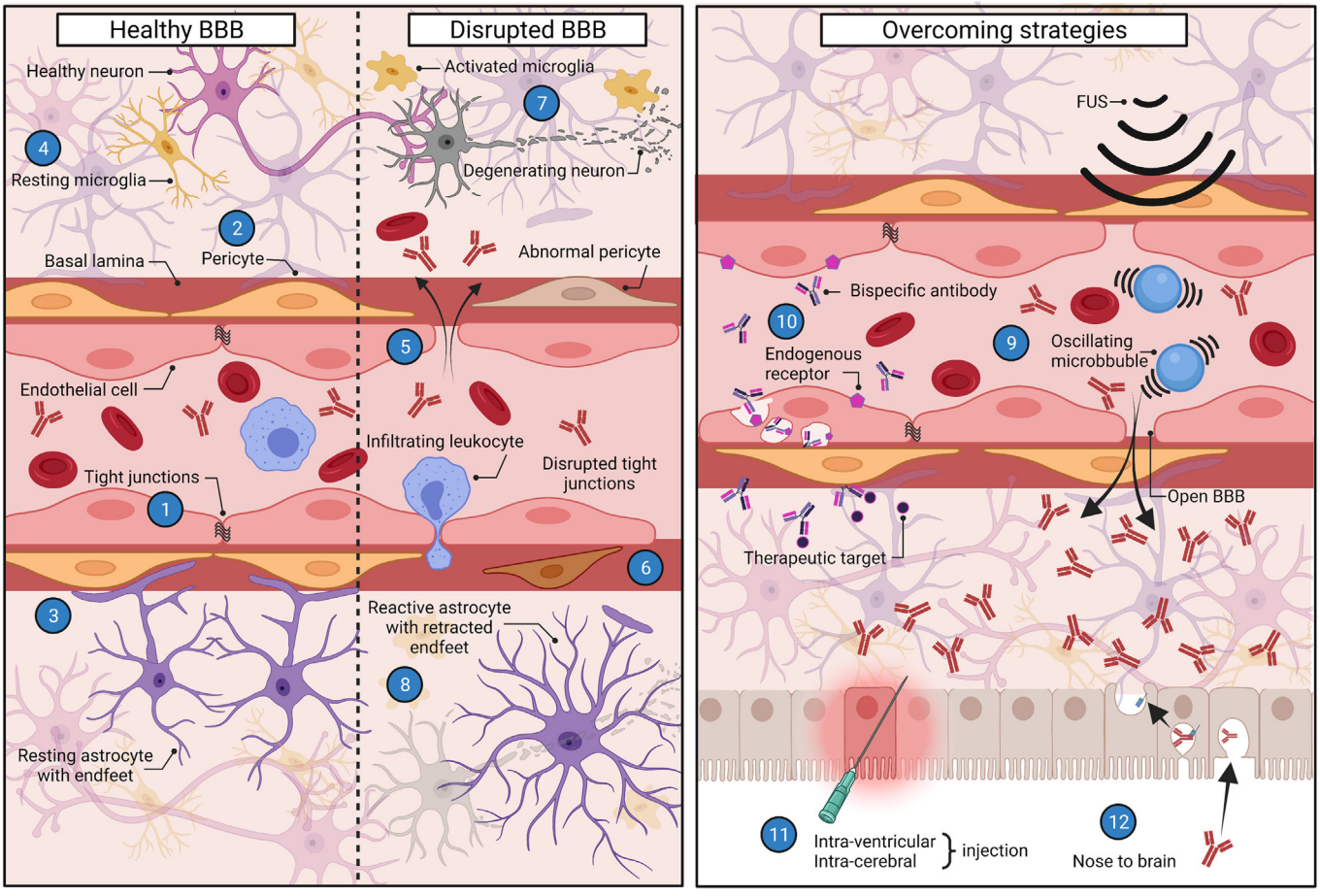

Fig. 3. Schematic representation of the blood-brain barrier (BBB) to antibodies in healthy and disease conditions, and the strategies developed to overcome this barrier.
A healthy BBB (left panel) is composed of endothelial cells that are tightly sealed by tight junctions (1) and prevent paracellular transport, hindering antibodies from reaching the CNS. Pericytes (2), which share the basal lamina with endothelial cells, help maintain the BBB and regulate blood flow through the capillaries. Astrocytes interact with BECs through their end-feet (3) and contribute to the maintenance of the BBB integrity. Resting microglia (4) are motile and highly ramified surveillant cells that are constantly scanning the brain environment to maintain its homeostasis. In pathological conditions (middle panel), the BBB becomes hyper-permeable to blood-born cells and molecules, including antibodies. Tight junctions between endothelial cells are disrupted (5), leading to a loss of endothelial cell polarity and uncontrolled paracellular transport. Pericytes are lost and detach from endothelial cells (6). Activated microglia (7) and reactive astrocytes (8) undergo molecular and morphological changes and adopt a pro-inflammatory phenotype. Studies suggest that BBB permeability increases with age, therefore increasing the passage of antibodies from the periphery to the CNS. Strategies to overcome the BBB (right panel) and increase antibodies distribution into the CNS were developed, including brain-focused ultrasound (FUS) combined with administration of microbubbles that open reversibly the BBB (9); bispecific antibodies (10) that target endogenous receptors expressed in the BBB and facilitate transcytosis; invasive, direct injection to the brain (11) via intra-cerebroventricular or intra-cerebral administrations; and nose-to-brain delivery (12), where antibodies are delivered to the nose and cross the olfactory epithelium, reaching the brain.

et al., 2007; Stamou, Grodzki, van Oostrum, Wollscheid, & Lein, 2018). FcRn in the brain is involved in reverse transcytosis of IgG across the BBB by a rapid efflux of IgG secreted in the brain to blood direction (Cooper et al., 2013; Schlachetzki et al., 2002). However, despite numerous studies in brain endothelial cells, the role of FcRn in IgG recycling and transcytosis from blood to brain has not yet been clearly demonstrated (Abuqayyas & Balthasar, 2013; Chen et al., 2014; Deane et al., 2005; Yip et al., 2014). For example, it was reported that systemically injected Ab in FcRn knockout mice presented similar plasma to brain ratio compared to wild-type mice (Garg & Balthasar, 2009). More recently, a study performed in brain endothelial-like cells derived from human induced pluripotent stem cells showed that transcytosis occurs independently of FcRn, as IgGs lacking human FcRn recognition presented the same permeability as a full-length control IgG (Ruano-Salguero & Lee, 2020). Even if several studies demonstrated that FcRn rapidly promotes efflux of therapeutic antibody and hamper their therapeutic potential, studies performed in Alzheimer's disease models suggest that FcRn can actually improve the therapeutic effect of antibodies targeting amyloid-β (Aβ) plaques by promoting the efflux of anti-Aβ antibody/Aβ immune complexes (Deane et al., 2005; Deane, Bell, Sagare, & Zlokovic, 2009). Intriguingly, studies performed in a transgenic AD mouse model suggested that FcRn expression decreases with age (Deane et al., 2005). The putative occurrence of such decrease in the human ageing brain suggests two scenarios: (i) Ab administered at the periphery would present a lower efflux ratio than described so far; or (ii) Ab/antigen complexes would be trapped in the CNS thus preventing any therapeutic effect induced by immunotherapy.

Disruptions in the BBB have been reported in several pathological conditions (Zlokovic, 2008), involving several cellular and molecular actors, like proteases (Yang & Rosenberg, 2011), ultimately resulting in the increased permeability of the BBB (Fig. 3), associated to the disruption of tight junctions (Gaillard, de Boer, & Breimer, 2003) and changes in pericytes (Bohannon et al., 2020). Studies also reported an age-associated increase in the BBB permeability, which implies a potential age- and disease-induced increase in the antibody uptake (Bell et al., 2010; Erickson & Banks, 2019; Popescu et al., 2009). Among the consequences of increased BBB permeability is the leakage of serum proteins including antibodies (Gray & Woulfe, 2015; Nelson, Sweeney, Sagare, & Zlokovic, 2016; Wenting et al., 2020; Zamudio et al., 2020) and the infiltration of peripheral cells such as T cells, macrophages, neutrophils, and red blood cells (Blair et al., 2015; Zamudio et al., 2020). These changes lead to the activation of microglia and astrocytes, and an inflammatory cascade exacerbating the condition and leading to a vicious cycle whereby the BBB disruption causes an inflammatory response, which in turn disrupts the integrity of the BBB (Chan-Ling et al., 2007; Oksanen et al., 2019; Tuppo & Arias, 2005). Studies on neurodegenerative diseases reported an enhanced uptake of drugs that usually are unable to cross the BBB (Kortekaas et al., 2005). For example, trastuzumab uptake increased by 18-fold in metastatic brain lesions once the tumour size was superior to 0.5 mm (Dijkers et al., 2010), which is enough to cause a disruption of the BBB (Ni, Chen, & Lu, 2018). Other antibodies have also been successfully delivered to ischemic brain regions following a stroke-induced BBB opening and showed significant therapeutic and prognostic results (Irving et al., 2005; Macrez et al., 2011; Rust et al., 2019). However, alterations in the BBB permeability in CNS disorders do not always favour an increased permeability for peripheral antibodies (Bien-Ly et al., 2015; Wahl et al., 2020).

Despite promising results in experimental models of CNS diseases, where peripherally administered antibodies were able to reduce symptoms (Atwal et al., 2011; Bard et al., 2000; Buttini et al., 2005; Masliah et al., 2011), there seems to be many difficulties to translate them in humans. Several clinical trials involving anti-Aβ mAbs were terminated after interim analysis indicating that systemically administered mAbs were unlikely to meet they primary endpoint (Blennow et al., 2012; Doody, et al., 2014; Ostrowitzki et al., 2017; Rinne et al., 2010; Salloway et al., 2021). One of the reasons why these mAbs failed in clinical trials is the need to administer large mAb doses to achieve a therapeutic effect in the CNS, which increases the risk of side effects. Recently, Aducanumab became the first anti-Aβ mAb to be approved by the FDA for the management of Alzheimer's disease. However, the effectiveness of this intravenously administered mAb is still controversial and its approval debatable due to its considerable side effects and unproven therapeutic potential (Mullard, 2021).

3.3.2.1. Blood-brain barrier: overcoming strategies. Since peripherally administrated mAbs that target the CNS have been facing lack of success, several strategies have been evaluated to overcome the BBB and are reviewed below.

Direct delivery into the CNS: Direct antibody delivery into the brain and therapeutic effects were successfully achieved after intracerebroventricular injection (Cheng et al., 2017; Du et al., 2020; Gros-Louis, Soucy, Lariviere, & Julien, 2010; Klyubin et al., 2005; Qiang et al., 2018; Thakker et al., 2009; Yadav et al., 2017), intracerebral injection (Brendza et al., 2005; Grossi et al., 2003; Lombardo et al., 2003; Wilcock et al., 2003) and convection-enhanced delivery (Nwagwu, Immidisetti, Bukanowska, Vogelbaum, & Carbonell, 2020; Shoji et al., 2016; Souweidane et al., 2018). Although potentially effective, such neurosurgical methods are technically challenging, highly invasive, and raise serious safety concerns (Cohen-Pfeffer et al., 2017; Pardridge, 2007). In addition, such techniques show limited diffusion and distribution capacities of the antibody in the brain parenchyma (Nagaraja et al., 2005; Salvatore et al., 2006; Yan et al., 1994).

Antibody engineering: In an attempt to increase the uptake of peripherally injected antibody by the CNS, bispecific antibodies have been developed. As their name imply, they are capable of simultaneously recognizing two different epitopes or antigens. To improve BBB crossing, one antibody-binding site would recognise an endogenous BBB receptor and therefore acting as a Trojan horse, while the other site would recognise a therapeutic target inside the CNS. The ideal target receptor for this receptor-mediated transcytosis across the BBB needs to be highly and specifically expressed on brain endothelial cells (Choudhari et al., 2021; Pardridge, 2017). Several receptors for receptor-mediated transcytosis across the BBB have been considered so far as antigen for bispecific antibodies, such as the insulin receptor, LRP1 and LRP2 CD98hc (Zuchero et al., 2016), but the transferrin receptor remains the most widely targeted delivery system (Tashima, 2020) with promising results in Alzheimer's disease models (Yu et al., 2014).

Artificial ways to reversibly increase the permeability of BBB: Other techniques can be used in combination to intravenously administered antibodies with the aim to reversibly increase BBB permeability. Recently, Lesniak and co-workers reported a high brain uptake of bevacizumab following intra-arterial administration and mannitol-induced BBB opening (Lesniak et al., 2019). This old technique is resurfacing for the delivery of antibody to the brain and is currently being tested in three clinical trials (NCT02861898, NCT02800486, NCT01269853).

A new technique, which uses brain-focused ultrasound (FUS) (Costa, Joaquim, Forlenza, Talib, & Gattaz, 2019) to sonicate systematically administered microbubbles, can reversibly and locally disrupt tight junctions (Shang, Wang, Liu, Zhang, & Xue, 2011; Sheikov, McDannold, Sharma, & Hynynen, 2008) and increase active transcytosis in the BBB (Sheikov et al., 2006), allowing antibody transport from the blood to specific brain regions. This method was found to be safe in non-human primates (McDannold, Arvanitis, Vykhodtseva, & Livingstone, 2012; Nwagwu et al., 2020), and recently in humans (Abrahao et al., 2019; Carpentier et al., 2016). In addition, brain-focused ultrasound was found to downregulate the efflux transporter, P-glycoprotein, in the targeted brain regions (Cho et al., 2016). Since 2004, when Sheikov and his colleagues demonstrated for the first time that brain-focused ultrasound allowed BBB permeabilization to molecules as large as IgG (Sheikov, McDannold, Vykhodtseva, Jolesz, & Hynynen, 2004), there has been several studies demonstrating the relevance of brain-focused ultrasound to improve the delivery of systematically administered

antibody into specific brain areas, particularly in tumours and Alzheimer's disease (Dubey et al., 2020; Janowicz, Leinenga, Gotz, & Nisbet, 2019; Jordao et al., 2010; Kinoshita, McDannold, Jolesz, & Hynynen, 2006; Kobus, Zervantonakis, Zhang, & McDannold, 2016; Leinenga, Bodea, Koh, Nisbet, & Gotz, 2020; Liu et al., 2016; Park, Zhang, Vykhodtseva, & McDannold, 2012; Raymond et al., 2008; Sheybani et al., 2021). Overall, brain-focused ultrasound seems to be a promising technique to deliver antibody to targeted areas in the CNS, but the secondary effects of this technique on the brain still need to be further assessed (Todd et al., 2020).

Bypassing the BBB: Finally, intracerebral drug delivery through the nasal interface ("Nose-to-brain") is a way to bypass the BBB. Drugs are deposited in the olfactory region of the nares where, after crossing the olfactory epithelium, they travel to the brain via mechanisms that still need to be fully determined. Disorders that may benefit from this novel route of administration include neurodegenerative diseases, post-traumatic stress disorder, pain and glioblastoma. In an *ex vivo* porcine model, endogenous Ig as well as various mAb were shown to diffuse through the nasal mucosa in an FcRn-dependent mechanism (see Section 3.1) (Ladel et al., 2018). In a murine model, administration of anti-Aβ mAb by intranasal route allowed a better intracerebral bioavailability than the intraperitoneal route (Chauhan & Chauhan, 2015), while maintaining its therapeutic properties (Cattepoel, Hanenberg, Kulic, & Nitsch, 2011). This has motivated the EU to launch the N2B-patch program in 2017 aiming at developing a mAb for the treatment of multiple sclerosis through the nose-to-brain route (https://cordis.europa.eu/project/id/721098). In 2021, another program, Bio2Brain, was also launched by the EU to enhance the bioavailability of mAb targeting the CNS (https://cordis.europa.eu/project/id/956977). Even if promising, this groundwork on nose-to-brain delivery still exposes mAb to the cellular and extracellular barriers of the nasal mucosa (see Section 2). Lately, a combination of brain-focused ultrasound and intranasal drug administration was found to enhance the delivery of antibodies to gliomas in mice (Ye, Yuan, Yue, Rubin, & Chen, 2021). Ferreira and her colleagues also showed that nanoparticles coated with a mAb targeting EGFR were successfully delivered to glioblastoma following intranasal administration (Ferreira et al., 2021). Further optimisation is needed to increase the retention time at the nasal surface and to enhance the penetration of the nasal epithelium.

4. Immune barrier and antibody clearance

Among the different types of cells engaged in the immune responses, macrophages are at the crossroad between innate and adaptive immunity. Tissue-resident macrophages are found in various tissues through the body, like Kupffer cells in the liver, microglia in the brain, osteoclasts in bones, or alveolar macrophages in the lungs. Some tissue-resident macrophages are known to have a major role in IgG transport, biodistribution or metabolism and may be considered as an immune barrier against recombinant antibodies.

Only antibody fragments (Waldmann, Strober, & Mogielnicki, 1972), which have low-molecular weight as compared to full-length IgG/mAb above the glomerular cut-off threshold can be filtered by the kidneys. Consequently, antibody clearance occurs mainly through intracellular catabolism, which includes upstream engulfment of antibody through non-specific pinocytosis, target-mediated or FcγR-mediated endocytosis (W. Wang, Wang, & Balthasar, 2008). This latter process is mediated through binding of IgG Fc region or immune-complexes to FcγR-expressing cells such as phagocytes from mononuclear phagocytic system, including notably Kupffer cells, Langherans cells in the skin and alveolar macrophages. This binding will result in antibody lysosomal proteolysis. Kupffer cells represent the largest population of tissue macrophages (Nguyen-Lefebvre & Horuzsko, 2015). They are mainly found in the lumen of hepatic sinusoids and exhibit endocytic activity against blood material entering the liver (Naito, Hasegawa, Ebe, & Yamamoto, 2004) including immune-complexes via FcγR-dependent uptake (Johansson et al., 2002). Similarly, there is evidence indicating that alveolar macrophages are involved in IgG catabolism. Indeed, Lombry et al. showed that clodronate-treated mice where alveolar macrophages were depleted, exhibited several-fold enhancement in systemic absorption of intratracheally-administered IgG (Lombry, Edwards, Preat, & Vanbever, 2004). Antibodies are supposed to cross slowly the epithelial-capillary barrier and consequently can remain within parenchyma for prolonged period as compared to small molecules, giving more time to resident phagocytes to engulf them. FcγR binding alone is not expected to have a major impact on IgG PK. Indeed, studies with FcγR-/- mice showed that FcγR-mediated clearance played only a minor role (Abuqayyas & Balthasar, 2012). However, in some circumstances antibody binding to FcγR may limit its bioavailability, for example: (i) when phagocyte infiltration is increased due to inflammatory or infectious triggers, (ii) when considering larger antibody structure, like ADC that tend to have a better binding to FcγR (Lux, Yu, Scanlan, & Nimmerjahn, 2013), and (iii) when considering engineered antibody with improved affinity to FcγR. Preclinical studies reported a contribution of Fc–FcγR interactions between ADCs and tumour-associated macrophages, suggesting that they can contribute to ADC processing through FcγR interaction and may induce off target cytotoxicity (Li et al., 2017). It has been showed that trastuzumab emtansine exhibited faster clearance in mice and humans compared to native antibody; this was suggested to be attributable to a better recognition and internalisation of antibody by FcγR-expressing phagocytes from the mononuclear phagocytic system (Burris 3rd et al., 2011; Leyland-Jones et al., 2003). Other factors like larger patient body mass or tumour burden, associated with increased mononuclear phagocytic system function, may accelerate antibody clearance (Bruno et al., 2005; Quartino et al., 2016). This should be put into perspective with the fact that for complex antibody-like structures like bispecific antibodies, rapid clearance was not attributable to target binding nor involved mononuclear phagocytic system but to binding with liver sinusoidal endothelial cells (Datta-Mannan et al., 2016). Overall, the exact contribution of FcγR-expressing phagocytes to antibody elimination remains not fully understood.

In addition, many immune cells express FcRn: myeloid lineage such as dendritic cells, monocytes/macrophages and neutrophils (Akilesh et al., 2007; Zhu et al., 2001) mainly express FcRn while it is detected at a lower level in lymphoid cells (T and B lymphocytes) (van Bilsen et al., 2010). In bone marrow chimera experiments using FcRn wild-type and knockout mice, Akilesh et al. showed that FcRn expression in bone marrow-derived cells plays a significant role in regulating IgG serum half-life and homeostasis (Akilesh et al., 2007). Nevertheless, the loss of FcRn expression in B cells and dendritic cells had no significant effect on IgG homeostasis, in mice. The role of macrophages in the recycling and homeostasis of IgG (Challa et al., 2019) may be more prominent as they express FcγR and the highest level of FcRn. Additional factors including antibody glycosylation, aggregation, and sensitivity to proteolysis, (Jiang et al., 2011) may be involved in phagocyte-mediated clearance and further studies are warranted to better understand and delineate the contribution of immune cells in antibody homeostasis.

4.1. Immune barrier: overcoming strategies

The dual role of FcR in IgG catabolism and PK/PD makes any modifications of IgG affinity to FcγR or FcRn susceptible to ultimately alter mAb efficacy. Thus, protein engineering in the Fc domain of IgG must be considered with caution, depending on the target localization and pathological context, which will profoundly affect the nature and the magnitude of the immune cell populations encountered by mAb. Interestingly, a recent study took the advantage of the phagocytic pathways to achieve therapeutic efficacy. Kasturirangan et al. showed that a bispecific/biparatopic antibody construct generated with two antibodies targeting different epitopes of IL-6 was much rapidly internalised and degraded by Kupffer cells as compared to parental antibodies. This

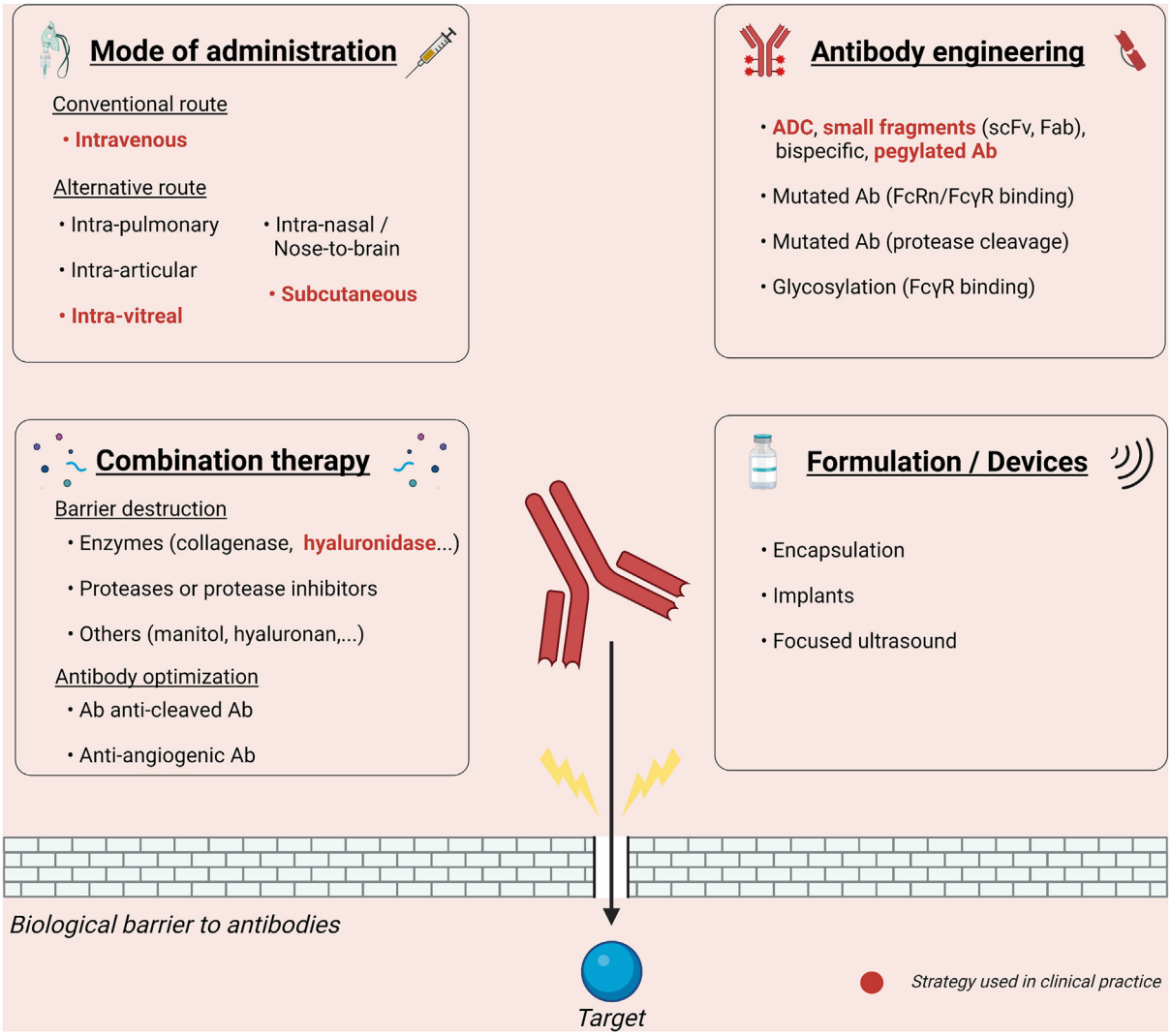

Fig. 4. Therapeutic strategies to overcome barriers to antibodies.
Depending on its location, different therapeutic strategies have been developed or are under consideration to help mAb reaching their target through the modulation of biological barriers. The choice of the mode of administration or specific tools and devices may allow a direct access to the target location. mAb engineering and/or formulation will help lowering the detrimental impact of barrier components. Finally, the administration of mAb in combination with a co-treatment will either destroy the barrier or improve mAb stability, favouring its access to its target.

describing novel approach will both promote neutralisation but also clearance of soluble targets (Kasturirangan et al., 2017).

5. Conclusion

In most cases, antibodies have to face several cellular and non-cellular barriers after entering the human body to reach their target antigens. The barriers that antibody may encounter depends on:

i) the delivery route. Although, the intravenous route may not be optimal to efficiently deliver antibody to specific organ or tissues, like the brain, lungs, gastro-intestinal tract, eye or the joints, exposing antibody to low-permeable barriers, antibody delivered by alternative routes are also confronted with cellular and non-cellular barriers, which should be considered during antibody-drug development.
ii) the antibody format/structure, which confers different partitioning properties in the different body compartment parts. Although IgG may benefit from FcRn uptake for transcytosis, their high molecular weight may be detrimental for diffusion across non-cellular barriers as compared to antibody fragments. More comparative studies addressing the impact of antibody format/structure on barrier crossing would be valuable but difficult to implement since the format/structure is also modifying antibody half-life.
iii) the pathological conditions and inter-individual variability response to disease. As oncology is a major medical application field for recombinant antibody, the impact of tumour stroma on antibody diffusion has constituted a thriving focus of research. In contrast other barriers, which can be altered in non-cancerous pathological conditions, such as proteases, mucus, biofilm have driven lower attention so far and should be taken into consideration to avoid antibody clinical failure.
iv) aging, which is certainly an under-appreciated parameter in antibody response. The present review briefly mentioned the impact of aging on the decreased function of organs, like the brain, as well as the significant decline in the efficacy of the immune system that may ultimately leads to age-associated diseases (Erdo, Denes, & de Lange, 2017; Owyong et al., 2018). The influence of ageing on the nature and magnitude of the biological barriers and response to antibodies has been poorly characterized so far and may certainly require more attention especially

Table 1
Approved strategies to overcome biologicals barriers for therapeutic antibodies

Indication	Generic name (trade name)	Sponsoring company	Antibody target	Antibody format	Combined molecule	Date of approval	Route of administration
HER2+ metastatic breast cancer	Herceptin	Roche	HER2	Full-length		2013	
Primary immunodeficiency	HYQVIA	Shire Pharmaceuticals	IgG replacement	Full-length		2013	
HER2+ early/metastatic breast cancer	Phesgo	Roche	HER2	Full-length	rHuPH20	2021	Subcutaneous
Non-Hodgkin lymphoma	Rituxan Hycela / Rituxan SC / MabThera SC	Roche	CD20	Full-length		2014	
Age-related macular degeneration	Avastin	Genentech	VEGF	Full-length	–	Off-label use	Intravitreal
	Eylea	Regeneron pharmaceuticals	VEGF/PlGF	Full-length		2011	
	Lucentis	Genentech	VEGF	Fab		2006	
Rhumatoid arthritis / Crohn disease / Psiorasis	Cimzia	UCB Pharma	TNF	Fab	PEG	2008	
Hodgkin lymphoma	Adcetris	Seagen (Seattle Genetics)	CD30	ADC, protease sensitive linker	MMAE	2011	
HER2+ metastatic breast cancer	Enhertu	Daiichi Sankyo	HER2	ADC, protease sensitive linker	DXd	2019	
Urotelial cancer	Padcev	Astellas	Nectin-4	ADC, protease sensitive linker	MMAE	2019	
Diffuse large B-cell lymphoma	Polivy	Roche	CD79b	ADC, protease sensitive linker	MMAE	2019	
Cervical cancer	Tivdak	Genmab	Tissue factor	ADC, protease sensitive linker	MMAE	2021	
Diffuse large B-cell lymphoma	Zynlonta	ADC therapeutics	CD19	ADC, protease sensitive linker	PBD	2021	
Acute lymphoblastic leukemia	Besponsa	Pfizer	CD22	ADC, pH-sensitive linker	Calicheamicin	2017	
Triple-negative breast cancer	Trodelvy	Immunomedics	TROP-2	ADC, pH-sensitive linker	SN38	2020	
Acute myeloid leukemia	Mylotarg	Pfizer	CD33	ADC, pH-sensitive linker	Calicheamicin	2017	Intraveinous

ADC, antibody-drug conjugate; DXd, topoisomerase I inhibitor; in.v, intravitreal; i.v, intravenous; MMAE, Monomethyl auristatin E; MMAF, Monomethyl auristatin F; PBD, pyrrolobenzodiazepine; PEG, polyethylene glycol; rHUPH20, hyaluronidase; SN38, irinotecan.

with the increased ageing of populations and the relevance of antibodies in the treatment of age-associated diseases.

As illustrated (Fig. 4), many strategies have been or can be investigated to overcome both natural and pathological barriers to improve antibody distribution and ultimately their therapeutic response. However, few of them have been materialised in the clinic successfully (Table 1). A better understanding of the consequences of each barrier/barrier component on antibody partitioning will be required to help develop antibodies with novel clinical applications or delivery routes.

Declaration of Competing Interest

YA, TB, JL, MG, AP, CDS, NJ, JPP, VGG, DL, SP, TS, have nothing to declare. NHV is co-founder and scientific expert for Cynbiose Respiratory. In the past two years, she received consultancy fees from Eli Lilly, Argenx, Novartis.

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