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**ANTIGEN BINDING MOLECULES SELECTIVE FOR CELLULAR FIBROBLAST ACTIVATION PROTEIN
(FAP) OVER SOLUBLE FAP**

1. FIELD OF THE INVENTION

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The present invention relates to antigen binding molecules that specifically bind cellular bound fibroblast activation protein (cFAP). The antigen binding molecules significantly discriminate between cFAP and one or more soluble variants of fibroblast activation protein (FAP), including the one or more soluble variants of FAP known to be present in body fluids
15 such as blood, plasma and serum. The antigen binding molecules comprise one or more antibodies and/or one or more antibody antigen binding domains or fragments, and, in particular, include one or more camelid single antigen binding domains, i.e. one or more V_HH sequences. The present invention further relates to nucleic acid molecules encoding the antigen binding molecules or a component domain thereof, as well as vectors comprising said
20 nucleic acid molecules. The invention also relates to a host cell comprising the vector(s) of the invention, as well as to methods for the production of an antigen binding molecule of the invention comprising culturing the host cell of the invention under suitable conditions and isolating the antigen binding molecule produced. Furthermore, the present invention relates to an antigen binding molecule obtainable by the method of the invention, to a composition
25 comprising at least one of an antigen binding molecule of the invention, the nucleic acid molecule of the invention, the vector of the invention, the host cell of the invention or the antigen binding molecule produced by the method of the invention. The present invention also relates to the use of an antigen binding molecule of the invention for detecting and targeting cFAP *in vivo* or *in vitro*, in particular selectively over one or more soluble variants of
30 FAP, for use as a therapeutic in the treatment of diseases characterized by cFAP or as a diagnostic agent.

2. BACKGROUND

Fibroblast activation protein (FAP), also known as fibroblast activation protein-alpha (FAP- α), is a member of the prolyl-specific serine protease family, having both enzymatic and signalling activities. FAP exists as a full length cellular bound protein (cFAP) of 760 amino acids (AA) as well as in soluble form in plasma, which is likely a number of different variants having differing truncated sequences. Thus, the term FAP without further specification can refer to both cFAP as well as any of the multiple truncated extracellular, soluble, non-membrane associated variants in human plasma, i.e. the soluble plasma variants of FAP (spFAP). It is believed that the cleavage starting position of spFAP is within approximately the first 40 N-terminal residues of cFAP, but it has yet to be reproducibly established. The sequence of at least one spFAP has been approximated by Lee et al., Blood 107(2006), 1397-1404 (SEQ NO: 2).

FAP is normally only expressed in injured tissue undergoing active repair. Thus, it has been recognized as a highly attractive therapeutic and diagnostic target. Except for during active tissue repair, it has been established to be rarely expressed in normal tissue and to be overexpressed in several disease states associated with extracellular remodelling, e.g. fibrosis, inflammatory disease, arthritis, atherosclerosis, autoimmune diseases, metabolic diseases and cancer; Fitzgerald and Weiner, Cancer Metastasis Rev. 39(2020), 783-803. For example, its (over)expression has been established in benign gynecological neoplasms such as endometriosis (WO 2023/198677) and myoma (Luo et al., Transl. Res. 163(2014), 232-241).

However, antigen-binding molecules targeting FAP such as the humanized monoclonal antibody sibrotuzumab (WO2002083171A2) and the antibody 4B9 (Waldhauer et al., MAbs. 13(2021), 1913791) have as yet failed to demonstrate clinically relevant therapeutic efficacy. The presents inventors have recognized that the lack of efficacy can be in part attributed to poor pharmacokinetics (PK) due to the sink effect caused by spFAP, i.e. current anti-FAP antibodies binding non-target spFAP, present in healthy individuals at an average concentration of 100ng/mL. Accordingly, binding molecules with selective, e.g. preferential or exclusive, binding to cFAP over spFAP would have significant utility for therapeutic and diagnostic use. In particular, such selective binders are desirable because their use would avoid off-target tissue trafficking (causing unintended side-effects), poor PK, and/or other side

effects that may be associated with locations where spFAP is present. Additionally, the use of such selective binders could improve the potential therapies of current anti-FAP molecules because they would not have to be dosed at higher levels to overcome the sink effect, and thereby lower dosages, costs and/or allowing more attractive dosing regimens.

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3. SUMMARY

Despite the potential significant overlap between cFAP and its soluble variants (e.g. as multiple potential variants existing in plasma, "spFAP"), as well as the issues known in the art associated with the development of antigen binding molecules capable of distinguishing such highly similar molecules, the inventors have surprisingly developed antigen binding molecules characterized by selectively binding to cFAP over soluble versions of FAP, in particular spFAP. The developed antigen binding molecules are the first in class of such molecules, and are in particular characterized by the activity of discriminating cFAP from one or more soluble variants, e.g. spFAP. The antigen binding molecules demonstrate a remarkably high specificity for cFAP, which molecules also selectively do not bind soluble variants e.g. as present in human blood (spFAP). Analysis of the identified antigen binding molecules also allowed the development of consensus structures that can impart the specific and selective binding to cFAP as defined herein. Thus, provided are antigen binding molecules and the use thereof as targeting agents and/or diagnostic and prognostic tools for the therapy (including the diagnosis, treatment or amelioration) of diseases characterized by cFAP expression.

The antigen binding molecules provided herein comprise or consist of one or more antibodies, antibody antigen-binding domains or antibody antigen-binding fragments, such as, in a non-limiting example, single domain antigen-binding fragments. The antigen binding molecules of the invention, as demonstrated by exemplary specific members of the family, specifically bind to cFAP. As is known in the art, FAP (also known as both fibroblast protein alpha (FAP- α) and prolyl endopeptidase FAP) is a type II transmembrane glycoprotein having 760 amino acids as represented by SEQ ID NO:1. Accordingly, the antigen-binding molecules of the invention specifically bind SEQ ID NO:1 as expressed on a cell surface. The cellular expression of cFAP can be endogenous (the cell expresses cFAP without recombinant manipulation) or the cell can be recombinantly modified to express cFAP. Thus, the antigen-binding molecules also

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specifically bind cells expressing cFAP, whether endogenously or recombinantly expressed. The cFAP specific and selective antigen-binding molecules of the invention are also interchangeably referenced herein as anti-cFAP molecules, anti-cFAP antibodies, anti-cFAP antibody fragments, anti-cFAP antibody-derived domains and the like.

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The antigen-binding molecules of the invention specifically bind the target antigen cFAP (e.g. as expressed on the surface of a cell-membrane), and selectively bind cFAP over (or do not bind) FAP in soluble form, e.g. low or no binding to sFAP as present in human plasma, serum or blood (spFAP, which may comprise one or more variants of sFAP). In a nonlimiting example, sFAP is represented by the amino acid sequence SEQ ID NO:2. Thus, the antigen-binding molecules discriminate cFAP as defined herein from FAP in soluble form, e.g. sFAP. The antigen-binding molecules also additionally or alternately discriminate cFAP as defined herein from soluble FAP variants as existing in plasma, serum or blood (spFAP), in particular human plasma, serum or blood. Because it is understood that that plasma, serum and blood contains spFAP, the selectivity of the antigen-binding molecules of the invention can be assessed by comparing the binding to cFAP relative to the binding of plasma, serum or blood known or expected to contain spFAP. Accordingly, the antigen binding molecules provided herein also preferentially and selectively bind target antigen cFAP in the presence of soluble forms of FAP, e.g. sFAP and/or spFAP. The antigen binding molecules provided herein also preferentially and selectively bind target antigen cFAP in the presence of plasma, serum or blood preferably human plasma, serum or blood. Without being limited to a specific explanation, it is believed that the anti-cFAP antigen binding molecules bind to an epitope of cFAP not present in soluble variants of FAP. As is understood in the art, "not present" as used in this context may mean that the particular amino acids forming the recognized epitope do not exist in the soluble variants, e.g. spFAP, or that the structure of the soluble variant(s) are altered relative to cFAP such that the three-dimensional epitope recognized by the antigen binding molecules of the invention is no longer presented or is otherwise not sterically accessible in the soluble variant(s). Thus, the anti-cFAP antigen-binding molecules and their antigen binding fragments may also bind to full-length FAP or partial sequences thereof when not expressed by a cell provided that the target epitope is present.

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As defined herein below, the terms “selectively bind” and “discriminates” are used interchangeably and indicate that the anti-cFAP antigen-binding molecules of the invention bind the specific antigenic target (i.e. FAP as endogenously or recombinantly expressed on the surface of a cell (cFAP)) with greater activity and/or specificity than they bind other antigens, in particular soluble forms of FAP as known in the art (sFAP) and/or as exists in human extracellular fluids, such as serum or plasma (spFAP). For example, as detailed herein below, the feature of selectively binding or discriminating a target antigen from/over a non-target antigen may be characterized by the anti-cFAP antigen-binding molecules (e.g. comprising or consisting of at least one antibody antigen-binding domain or region) having selective binding over one or more soluble variants of FAP, such as present *in vivo*, e.g. in plasma, serum, blood, tissue or body fluids, preferably human plasma, human serum, human blood, human tissue or human body fluids.

As disclosed herein, the specific and selective anti-cFAP antigen-binding molecules and antigen-binding fragments discriminate cFAP from one or more soluble variants thereof. Accordingly, it is most preferred that the specific and selective anti-cFAP antigen-binding molecules and antigen-binding fragments do not substantially bind to SEQ ID NO:2 or spFAP as existing in plasma, serum or blood. As understood in the art, because plasma, serum and blood, in particular human plasma, serum and blood, are known to contain spFAP, it is not necessary to isolate spFAP or any particular soluble FAP variant from the plasma, serum or blood. Thus it is also most preferred that the specific and selective anti-cFAP antigen-binding molecules and antigen-binding fragments do not exhibit significant binding activity to plasma, serum, or blood, in particular human plasma, serum or blood.

Selective binding for cFAP over one or more soluble variants, e.g. SEQ ID NO:2 and/or spFAP in blood, can be established by any assay or combination of assays known in the art or described herein such as wherein the binding of the specific and selective anti-cFAP antigen-binding molecule to cFAP is compared to its binding to one or more soluble variants sFAP and/or wherein a competition assay is performed by comparing binding to cFAP in the presence and absence of one or more soluble FAP variants, or in the presence and absence of plasma, serum or blood known or believed to contain soluble FAP, e.g. spFAP.

As a non limiting example, the specific and selective binding to cFAP over one or more soluble FAP variants, e.g. as known to exist in plasma, serum, blood or tissue (spFAP) can be assessed by measuring the EC₅₀ for binding of the specific and selective anti-cFAP antigen-binding molecules for binding to cFAP and comparing it to the EC₅₀ for binding to one or more soluble variants. The assays to determine specific and selective binding, e.g. by comparing the EC₅₀ for binding to cFAP and spFAP, are not limited and any suitable assay known in the art or described herein may be used. The assays used for the comparative studies, can be calibrated by the used of controls, e.g. assay conditions adjusted so that standard results are obtained for control molecules such as known anti-FAP molecules sibrotuzumab and/or 4B9. For example, it is preferred that the assay used to determine the EC₅₀ for binding to soluble FAP, e.g. spFAP, is performed under conditions where the EC₅₀ of sibrotuzumab or 4B9 for binding to spFAP is less than 1 nM or less than 10 nM, respectively.

An exemplary assay for the determination of the EC₅₀ of binding to cFAP is the use of flow cytometry. It is preferred that assay conditions are adjusted such that the EC₅₀ value for binding to cFAP is less than 5nM. It is further preferred that assay conditions are adjusted such that (a) the EC₅₀ of binding to said cFAP is within at least 1-fold of the EC₅₀ of sibrotuzumab or 4B9 as assessed under the same conditions; and/or (b) the maximum binding to said cFAP is within 1-fold of the maximum binding of sibrotuzumab or 4B9 as assessed under the same conditions.

Where the assays for binding to cFAP and soluble FAP, e.g. spFAP, are calibrated as above, e.g. adjusted to arrive at the preferred values for, or relative to, the control molecules such as sibrotuzumab or 4B9, specific and selective binding can be identified by an EC₅₀ value for binding to cFAP of less than 5 nM and an EC₅₀ binding to one or more soluble variants, e.g. spFAP, that is at least 20-fold weaker. The specific and selective binding to cFAP by antigen-binding molecules of the invention can also be identified by the molecules exhibiting one or more of the following cFAP binding properties as determined by flow cytometry: (a) an apparent K_d of binding to cFAP of less than 2nM; (b) an EC₅₀ of binding to cFAP of less than 2 nM; (c) an EC₅₀ of binding to said cFAP that is within at least 1-fold of the EC₅₀ of sibrotuzumab or 4B9 assessed under the same conditions; and (d) a maximum binding to cFAP that is within 1-fold of sibrotuzumab or 4B9 assessed under the same conditions.

Specific and selective binding may also be identified or established by determining (i) the EC_{50} and/or TOP (MFI) of the antigen-binding molecule to cFAP, such as recombinantly expressed on the surface of HEK 293F cells, and (ii) the EC_{50} of said molecule for binding to one or more soluble FAP, e.g. spFAP. In a non-limiting example, the anti-cFAP antigen-binding molecule of the invention is specific and selective for cFAP (over sFAP) where (i) the EC_{50} and TOP (MFI) for binding to FAP as expressed on the surface of a cell (e.g. SEQ ID NO:1 recombinantly expressed on the surface of HEK 293 cells) is at least 60% of the EC_{50} and TOP (MFI) of sibrotuzumab when assessed under the same conditions; and (ii) the EC_{50} of said molecule for binding to human undiluted plasma containing spFAP at a concentration of at least 80 ng/mL is (a) weaker than 40nM, (b) not detectable, or (c) incalculable when assessed in an assay having a control EC_{50} for sibrotuzumab or 4B9 binding to said undiluted plasma under the same conditions of below 1nM or below 10nM, respectively.

In a further non-limiting example, the specific and selective binding activity of the anti-cFAP antigen-binding molecule of the invention can be determined in a competitive binding experiment such as the detection and comparison of binding activity for cFAP in the presence or absence of spFAP, e.g. in the presence or absence of plasma, serum, tissue or blood. For example, the antigen binding molecule of the invention is specific and selective for cFAP (over one or more soluble variants of FAP, such as spFAP) where the ratio of binding to cFAP in the presence of undiluted human plasma known or believed to contain spFAP, e.g. at concentration of at least 80ng/mL, relative to the binding in the absence of said undiluted human plasma is at least 80% at an antigen-binding molecule concentration of 0.8nM, at least 60% at an antigen-binding molecule concentration of 0.16 nM, or at least 50% at an antigen-binding molecule concentration of 0.0032 nM. It is preferred that the comparative assay in this context exhibits under the same conditions a ratio of binding for 4B9 or sibrotuzumab to cFAP in the presence of said undiluted human plasma relative to the binding in the absence of said undiluted human plasma of less than 10% or less than 25%, respectively, wherein the 4B9 or sibrotuzumab concentration is less than or equal to 0.8nM and greater than or equal to 0.0032 nM, e.g. 0.8 nM, 0.016 nM, or 0.0032 nM.

Any cFAP known in the art can be used in the comparative assays, but it is preferred that the cFAP has the amino acid sequence of SEQ ID NO:1. As disclosed herein, cFAP is also membrane bound and, thus, must be expressed on the surface of a cell or a cell-membrane to allow assessment of specific and selective binding. cFAP may be expressed in any suitable cell as known in the art or described herein. In a non-limiting example, the cFAP having the amino acid sequence SEQ ID NO:1 is recombinantly expressed on the surface of a HEK293F cell. Similarly, any known soluble variant of FAP can be used. As explained herein, multiple variants of soluble FAP are believed to exist in plasma, serum, blood and tissue, and differ from cFAP by having different truncated amino acid sequences. Thus, the use of any one or more soluble variant is contemplated. In a non-limiting example, the soluble FAP variant for use in the comparative assessment is sFAP having the amino acid sequence SEQ ID NO:2. Alternately or additionally, the soluble variant can be the one or more soluble FAP variant present in plasma, serum, blood or tissue, i.e. spFAP. It is contemplated that the spFAP need not necessarily be isolated from the plasma, serum, blood or tissue, but that serum, plasma, blood or tissue can be used (diluted or undiluted) as spFAP.

The specific and selective anti-cFAP antigen-binding molecules comprise or consist of one or more antibodies, antibody antigen-binding domains and/or antibody antigen-binding fragments, i.e. comprise or consist of one or more antibodies and/or domains and fragments derived therefrom that provide the capacity for antigen binding as known in the art. Accordingly, the anti-cFAP antigen-binding molecules of the invention may comprise or consist of one or more antibodies or antibody antigen-binding domains or fragments including, but not limited to Fv domains (i.e., paired heavy and light chain variable domains, such as Fab, Fab', F(ab')₂, and Fv fragments as well as recombinant constructs such as single-chain Fv domains (scFvs)) as well as antigen binding fragments and domains that comprise a single, unpaired heavy or light chain variable domain as known in the art that retain the ability to specifically and selectively bind target antigen as defined herein, including but not limited to single domain antibodies (also referenced in the art as sdAbs, dAbs, and/or nanobodies) and V_HH domains based on the heavy chains of camelids. It is preferred that the anti-cFAP antigen-binding molecules of the invention comprise or consist of one or more V_HH domains.

Antigen binding specificity is determined by the portions of the antibody antigen-binding domains and fragments that contact the ligand. These are known as the complementarity determining regions (CDRs). The CDRs are the most variable part of antibodies and contribute to their diversity. As is well understood, there are three CDR regions CDR1, CDR2 and CDR3 in each heavy and light chain variable domain, embedded between four framework regions (FW) according to the general pattern framework FW1-CDR1-FW2-CDR2-FW3-CDR3-FW4. Where the antigen-binding molecule is a single domain antibody, the antigen binding specificity is determined by a single set of three CDRs, e.g. the three CDRs of a heavy or light chain variable domain. For example, in a V_HH, the antigen-binding specificity is determined by the V_HH CDR1, CDR2 and CDR3.

The boundaries and lengths of the individual CDRs are subject to different classification and numbering systems as known in the art, including but not limited to those referenced as the Kabat, Chothia and IMGT systems as described in, e.g. Kabat et al., in "Sequences of Proteins of Immunological Interest," 5th Edition, U.S. Department of Health and Human Services, 1992; Chothia et al., J. Mol. Biol. 196(1987), 901, and Lefranc et al., Dev Comp Immunol 27(2003), 55-77, respectively. Unless otherwise indicated the CDR domains as referenced herein are defined according to IMGT.

The term "comprising", as used herein, denotes that further sequences/components can be included in addition to the specifically recited sequences and/or components. In those embodiments where the antigen-binding molecules comprise more than one or more antibody antigen-binding domains or fragments, e.g. one or more V_HH antibodies, additional amino acids and/or functional domains of the molecule of the invention can be present at either the N-terminal end, the C-terminal end, or both of the antibody antigen-binding domain(s). Additional sequences can include, without limitation sequences introduced for purification or detection, or sequences introduced to impart further activity (e.g. Fc region functionality/activity such as Fc receptor binding) as known in the art. Furthermore, where individual sequences "comprise" the recited sequence, they also can include additional amino acids at either the N-terminal end, or the C-terminal end, or both.

The specific and selective anti-cFAP antigen-binding molecules according to the invention may comprise an Fc region. As used herein, the term “Fc region” refers to the C-terminal region of an immunoglobulin heavy chain containing at least a portion of the constant region capable of binding one or more Fc receptors, preferably one or more FcγR receptors. The term includes native sequence Fc regions and variant Fc regions. As is understood in the art, the term “Fc region” and analogous phrases mean the polypeptide comprising the constant region of an antibody excluding the first constant region immunoglobulin domain and, in some cases, part of the hinge. Thus “Fc region” and analogous terms/phrases typically refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM, the Fc region may include the J chain. For IgG, the Fc region comprises immunoglobulin domains Cy2 and Cy3 and the lower hinge region between Cy1 and Cy2. Although the boundaries of the Fc region may vary, the human IgG heavy chain Fc region is usually defined to include residues C226 or P230 to the carboxyl-terminus. It is further understood that depending on production process the sequence of the Fc region may be altered by post-translational modifications, in particular, the terminal residues. In a non-limiting example, it is understood that depending on the production process, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Accordingly, the antigen-binding molecules as disclosed herein may comprise a variant of any of the foregoing Fc regions. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also known as the EU index, e.g. as described in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991.

The Fc region of the antigen-binding molecules of the present invention may be a modified Fc region relative to the native (wild-type) Fc region sequence, e.g. exhibiting increased binding to one or more FcγR receptors and/or exhibiting increased ADCC or ADCP activity. Such modifications are well known in the art and include, but are not limited to the mutation S239D/A303L/I332E according to EU numbering (also known as the DEL modification) and afucosylation modifications. Accordingly, it is preferred that the Fc region exhibits increased activity relative to the native (wild-type) sequence. A “native (wild-type) sequence Fc region” comprises an amino acid sequence identical to the amino acid sequence of an Fc region found

in nature. Because the use of the specific and selective anti-cFAP antigen binding molecules in humans is preferred, it is contemplated that the Fc region is a human Fc region or a variant/modified human Fc region. As described herein, the variant/modified Fc regions may exhibit increased binding activity to one or more FcγR receptors and/or increased ADCC or ADCP activity relative to a native or wild-type Fc region. Thus, where the specific and selective anti-cFAP antigen binding molecule of the invention comprises a modified or variant Fc region, it is preferred that the reference antigen-binding molecule for determination of increased Fc-region activity as described herein comprises the same antigen-binding region/domain/fragment as the test molecule and the native Fc region sequence of human IgG1, having the amino acid sequence SEQ ID NO:22.

It is further contemplated that the Fc region of the disclosed antigen-binding molecules (where present) can include further modifications known in the art to modulate antibody Fc interaction with Fc ligands other than FcγRs. Such ligands include FcRn and Fc receptor homologs including but not limited to FcRH1, FcRH2, FcRH3, FcRH4, FcRH5, and FcRH6; see, e.g. Davis et al., 2002, Immunol. Reviews 190:123-136.

As set forth in the examples, the inventors have developed antibodies specific and selective for FAP as expressed on the surface of a cell, i.e. cFAP, in particular over soluble variants of FAP, such as soluble recombinant FAP (sFAP) having the amino acid sequence of SEQ ID NO:2 and/or the soluble variants existing in plasma, serum, tissue or blood (spFAP). Analysis of working exemplary embodiments of the invention further allowed the inventors to identify exemplary consensus sequences of V_HH CDRs as well as complete V_HH sequences that can impart the identified functional features of the specific and selective anti-cFAP antigen-binding molecules of the invention. The analysis proceeded from the understanding in the art that certain CDR/variable domain residues are predominantly responsible for antibody binding activity, while the remaining residues have less of an impact. Therefore, it is known that amino acid residues within the CDR and/or variable domain regions can be exchanged without necessarily leading to a (significant) loss of function. That is, it is known in the art that certain amino acid residues of the CDR and/or variable regions can be exchanged and sequence variants maintaining the desired functional properties be readily identified.

Accordingly, exemplary anti-cFAP antigen-binding molecules of the invention molecules comprising or consisting of one or more V_HH comprising

- (i) a CDR1 having the sequence of X₁X₂TX₃X₄X₅YAX₆G (SEQ ID NO: 3), wherein X₁ is A, G or S; wherein X₂ is P, R or G; wherein X₃ is K, F, R or N; wherein X₄ is S, R or G; wherein X₅ is T, S or N; and wherein X₆ is M, I or L;
- (ii) a CDR2 having the sequence of X₁I₂WX₃X₄X₅X₆TX₇YX₈DSVKG (SEQ ID NO:4), wherein X₁ is A or V; wherein X₂ is N, W or S; wherein X₃ is S or A; wherein X₄ is G or N; wherein X₅ is T or G; wherein X₆ is I, L, S, T or V; wherein X₇ is S, Q or N; and wherein X₈ is S or T; and/or
- (iii) a CDR 3having the sequence of AADX₁DFRTVGSRPSY (SEQ ID NO:5), wherein X₁ is R, S or K.

The above defined exemplary anti-cFAP antigen-binding molecules include molecules comprising or consisting of one or more V_HH comprising

- (i) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VINWSGTLTSYADSVKG (SEQ ID NO:8) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (ii) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VINWAGTLTSYADSVKG (SEQ ID NO:9) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (iii) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VISWSGTLTSYADSVKG (SEQ ID NO:10) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (iv) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTQYTDSVKG (SEQ ID NO:11) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (v) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTNYTDSVKG (SEQ ID NO:12) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14); or
- (vi) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTNYADSVKG (SEQ ID NO:13) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14).

The above-defined anti-cFAP antigen-binding molecules of the invention further include molecules comprising or consisting of one or more of the amino acid sequences

- (i) QVQLVESGGGLVQAGDSLSCVASAPTRSTYAMGWFRQAPGKEREFVAAVINWSGTLTSYADS
5 VKGRFTISRDNKNTVFLQMNSLKPDDTAVYYCAADRDFRTVGSRPSYWGGTPTVTSS
("ab1", SEQ ID NO:15);
- (ii) QVQLVESGGGLVEPGDSLRLSCAASGRTFSSYAIGWFRQAPGKEREFVAAISWSGGTTNYTDSV
KGRFTISRDNKNTVYLQMNSLKPDDTAVYYCAADRDFRTVGSRPSYWSKGTRVTSS ("ab3",
SEQ ID NO:16);
- 10 (iii) QVQLVESGGGLVQPGGSLRLSCSASAPTRSTYAMGWFRQAPGKEREFASVISWSGTLTSYADSV
KGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGGTTVTSS
("ab1z12", SEQ ID NO:17);
- (iv) QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISWSGGTTQYTDSV
KGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGGTTVTSS
15 ("ab3z7", SEQ ID NO:18);
- (v) QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISWSGGTTNYADSV
KGRFTISRDNKNTLYLQMNSLRPEDTAVYYCAADRDFRTVGSRPSYWGGTTVTSS
("ab3Z10", SEQ ID NO:19).

- 20 The anti-cFAP antigen-binding molecules of the invention may further comprise one or more variants of the V_HH CDRs and or complete sequences as defined herein. As used herein, the terms variant CDR and/or variant sequence indicate functional variants, i.e. having amino acid sequences that differ from the reference amino acid sequence but which differing sequence exhibits or maintains the same functional activity as the reference sequence. Specifically, the
- 25 exemplary antigen-binding molecules of the invention comprising one or more variant V_HH CDRs and/or variant V_HH sequences as indicated throughout this disclosure exhibit specific and selective/discriminative binding for cFAP as described herein.

- The amino acid sequences of the variant CDRs and/or sequences recited herein are defined
- 30 by a reference to an amino acid sequence with an amino acid substitution at one or more positions. As used herein, the term "substitution" refers to the replacement of an amino acid with another amino acid. Thus, the total number of amino acids remains the same. The

deletion of an amino acid at one position and the introduction of one (or more) amino acid(s) at a different position is explicitly not encompassed by the term "substitution."

With respect to variant CDRs, the modifications may occur within one or more CDR so long as the modification does not substantially reduce or otherwise modify the desired functional property of the reference antigen-binding molecule. Accordingly, in certain embodiments the CDRs as provided herein may be unaltered, or may be variants having no more than one, two or three amino acid substitutions relative to SEQ ID NO:6 to 14, which variants exhibit specific selective binding for cFAP as defined herein. In other embodiments, the antigen binding molecule of the invention may comprise one or more V_HH domains/antibodies having the amino acid sequence of SEQ ID NO:15 to 19 or an amino acid sequence having at least 80%, at least 85%, at least 90%, or at least 95% sequence identity thereto which molecule exhibits specific and selective binding for cFAP over one or more soluble variants, e.g. spFAP, as defined herein. In certain embodiments, the antigen binding molecule of the invention comprises one or more of amino acid sequences SEQ ID NO:15 to 19.

It is preferred that the amino acid substitution(s) be conservative residue substitution(s). The term "conservative amino acid substitution" is well known in the art and refers to the replacement of an amino acid with a different amino acid having similar biophysical properties. As used herein, the groupings of amino acids having similar biophysical properties are

- (a) the nonpolar, hydrophobic amino acids consisting of glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), and methionine (Met);
- (b) the polar, neutral amino acids consisting of serine (Ser), threonine (Thr), asparagine (Asn), and glutamine (Gln);
- (c) the positively charged, basic amino acids consisting of arginine (Arg), lysine (Lys), and Histidine (His); and
- (d) the negatively charged, acidic amino acids consisting of aspartic acid (Asp) and glutamic acid (Glu).

Thus, a conservative substitution is a substitution of a residue with another from its same group, i.e. (i) the substitution of a nonpolar, hydrophobic amino acid of group (a) with another

amino acid of group (a); (ii) the substitution of a polar, neutral amino acid of group (b) with another amino acid of group (b); the substitution of a positively charged, basic amino acid of group (c) with another amino acid of group (c); and/or the substitution of a negatively charged, acidic amino acid of group (d) with another amino acid of group (d). It is appreciated that the amino acids Cys and Pro are not included in the above groupings and/or listing of conservative substitutions because, as well known in the art, these residues are not suitable as general substituents. Where the residue Cys or Pro is to be substituted, as used herein a conservative substitution for Cys is with Ser or Ala, and for Pro is with Ala.

As used herein, the term selectively binds and/or discriminate(s) over and analogous terms with respect to two antigens, e.g. the antigen-binding molecule or antigen binding domain/fragment selectively binds or discriminates cFAP from/over one or more soluble variants, e.g. as existing in plasma, serum, tissue or blood (spFAP) indicates that the antigen-binding molecule or antigen-binding domain/fragment specifically binds to the target antigen cFAP but does not specifically bind to the non-target antigen, i.e. soluble variants thereof, e.g. spFAP as existing *in vivo* in plasma, serum, tissue, blood or body fluids. Accordingly, the terms selectively binds, discriminate(s) and analogous terms as used herein means that the antibody or antigen binding fragment “does not specifically bind”/“does not significantly bind” (which are used interchangeably) to the non-target antigen. It is well known in the art that the terms “specifically bind” and “does not significantly bind” designate the degree to which an antigen-binding molecule discriminates between two antigens. This is because it is known that no antigen-binding molecule (in particular comprising antibody derived antigen binding domains or fragments) has absolute specificity, in the sense that it will react with only one epitope whatever the conditions. That is, where other (non-target) antigens are present, an antigen binding molecule, domain or fragment can react to some extent with similar epitopes on these other (non-target) antigens. However, the affinity of the specific antigen binding molecule, domain or fragment for its target epitope/antigen is significantly greater than its affinity for related epitopes. This difference in affinity is used to establish assay conditions, under which the antigen binding molecule binds almost exclusively to a specific epitope. In this respect, the binding (or non-binding) of an antigen binding molecule to an antigen are not understood as absolutes. That is, the anti-cFAP antigen-binding molecules of the invention may exhibit some (residual) binding activity for other (non-)targets, but at significantly reduced levels relative to

the binding activity for FAP as expressed on the surface of a cell (cFAP). The feature of discriminating a target antigen from/over a non-target antigen may be characterized by exemplary assays as known in the art or as described herein above. In some embodiments, the anti-cFAP antigen-binding molecules of the invention may exhibit no detectable binding to the non-target antigens.

As has been detailed herein, it is preferred that the comparison to establish the features of discrimination, i.e. "specific binding", "not specifically binding", "selective binding" and/or "discrimination" with respect to the target an non-target antigens is determined using the same experimental protocol and same experimental conditions (e.g. same binding assay, concentration/density of test/assay antibody or antigen-binding fragment, antigen concentration/density/flow rate, etc.).

The binding properties of the anti-cFAP antigen-binding molecules may be established by any suitable method known in the art and/or as described herein that allows quantification of binding parameters, and, in particular, allowing their quantitative comparison. Methods for analysing the binding specificity and binding parameters of antigen-biding molecules are described, e.g. in Harlow & Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in Harlow & Lane (1999), Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press. Non-limiting examples of suitable studies include binding studies and blocking/competition studies carried out by methods such as e.g. IHC, IF, Flow cytometry analysis, FACS analysis, flow cytometric titration analysis (FACS titration), surface plasmon resonance (SPR, e.g. with BIAcore®), isothermal titration calorimetry (ITC), fluorescence titration, or by radiolabelled ligand binding assays. Further methods include e.g. western blots, ELISA (including competition ELISA)-, RIA-, ECL-, IRMA-tests, as well as physiological assays, like cytotoxic assays.

The anti-cFAP antigen-biding molecules of the invention may be produced by any technique described herein and/or as known in the art. For example, the molecules may be prepared, expressed, created or isolated by recombinant means, such as expressed using a recombinant expression vector transfected into a host cell, or prepared, expressed, created or isolated by any other means that involves splicing of encoding gene sequences into other DNA sequences.

Accordingly, provided are polynucleotides encoding the anti-cFAP antigen-binding molecules of the invention, and/or encoding their component domains or fragments, as well as vectors comprising such polynucleotides. The vectors need not necessarily be expression vectors, but may be vectors allowing the reproduction of the vector and, thus, the duplication of the polynucleotide sequences of the invention, e.g., by culture of a host cell comprising the vector. The vectors may also be suitable to allow the recombinant manipulation of the polynucleotide sequences of the invention as is known in the art. In preferred embodiments, the vectors are expression vectors comprising polynucleotides encoding the anti-cFAP antigen-binding molecules as disclosed herein, that when introduced into suitable prokaryotic or eukaryotic cells according to standard methods known in the art, result in the expression of the molecules of the invention or component fragment or domains thereof. The host cell of the invention may be a directly engineered cell, i.e. a cell having undergone direct transfection with a vector or polynucleotide as disclosed herein, or may be a daughter cell or progeny of the cell directly transfected. Thus, provided are methods for producing anti-cFAP antigen-binding molecules by culturing a host cell comprising a polynucleotide encoding the molecule or a component domain or fragment thereof (e.g., as contained in an expression vector). The methods further comprise recovering and isolating the expressed molecule or component domain or fragment thereof from the culture (e.g. from the cell fraction and/or the culture medium) using standard protein purification methods. Thus, the invention also provides for specific and selective anti-cFAP antigen-binding molecules obtainable by the methods disclosed herein.

The expression from host cells and/or their progeny is achieved by introducing one or more expression vectors encoding molecules of the invention into a host cell by standard techniques. The introduction of such expression vectors is known in the art and referenced herein as transfection, or transformation and encompasses a wide variety of standard techniques commonly used for the introduction of exogenous DNA into a eukaryotic or prokaryotic host cell. Non-limiting examples of suitable transfection methods include chemical, (e.g. Lipofection, calcium-phosphate precipitation, and DEAE-dextran), physical (e.g. microinjection, gene gun), viral (e.g. viral vectors such as retrovirus, lentivirus, adenovirus), non-viral-vectors (plasmid vectors, transfection). Non-limiting examples of suitable transformation methods include chemical, physical, or bacteriophage mediated.

Although it is possible to express the molecules of the invention in either prokaryotic or eukaryotic host cells, expression of molecules comprising antibody derived domains and fragment (such as V_HH antibodies) in eukaryotic cells is preferable, and most preferable is expression in mammalian host cells, because such eukaryotic cells (and in particular
5 mammalian cells) are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active molecule. Non-limiting examples of mammalian host cells for expressing the molecules of the invention include Chinese Hamster Ovary (CHO cells), NSO myeloma cells, COS cells, and SP2 cells.

10 The recombinant anti-cFAP antigen binding molecules and/or their antigen binding domains/fragments may have variable and constant regions (if present) derived from the germline immunoglobulin sequences of the species from which they are isolated after standard immunization and selection procedures known in the art, e.g. may comprise the germline immunoglobulin sequences of an alpaca. However, the sequences can be subjected
15 to *in vitro* mutagenesis wherein, in particular, the CDR sequences are combined with FW sequences from another species, e.g. from a human as is known in the process of humanization. Therefore, the amino acid sequences of the molecules of the invention disclosed herein can be sequences that while derived from and related to germline heavy or light chain sequences may not naturally exist within any endogenous antibody germline
20 repertoire *in vivo*.

The anti-cFAP antigen-binding molecules of the invention are envisioned as therapeutic and/or diagnostic tools, e.g. for use in therapy for the treatment of diseases characterized by the overexpression of cFAP. As used herein the term "treatment" does not imply complete
25 eradication of any disease or cause of a disease, but also encompasses the amelioration of any disease parameter or symptom either permanently or temporarily. In a non-limiting exemplary embodiment, the molecules of the invention can serve as targeting moieties in the diagnostic and therapeutic uses disclosed herein. In this context the molecules of the invention may be conjugated to a therapeutic or diagnostic moiety as known in the art or
30 described herein, e.g. a cytotoxin, radioisotope or other molecule having activity as a therapeutic or diagnostic moiety as known in the art.

The molecules of the invention may be directly conjugated to the diagnostic or therapeutic moiety or may be indirectly conjugated, e.g. by the use of a linker. The selection of the appropriate linker is made according to standards well known in the art. As is recognized, appropriate linkers can be selected for features such as stability and biocompatibility. Linkers can also be designed and/or selected so as to maintain the structural integrity and binding affinity of the antibody while facilitating the targeted delivery of the conjugated moiety, e.g. radiolabeled isotopes or therapeutics. Suitable linkers may include, but are not limited to, maleimide-thiol linkages, hydrazone bonds, or click-chemistry-based constructs that allow site-specific conjugation and controlled release. In certain embodiments, the linker may incorporate a cleavable functionality, such as a pH-sensitive bond or an enzyme-cleavable group, to ensure the selective release of the radioligand in the tumor microenvironment or at the site of disease.

In the context of in-vivo diagnostics, the molecules of the invention may, in a non-limiting example, be conjugated to a radioisotope, radiotracer, radionucleotide, luminescent probe, or fluorescent probe as known in the art (e.g., either directly or indirectly through covalent linkers, DOTA, or similar). Known radioisotopes that can be used in the context of diagnostic or therapeutic indications disclosed herein include but are not limited to: ^{18}F , ^{18}F -FAC, ^{32}P , ^{33}P , ^{45}Ti , ^{47}Sc , ^{52}Fe , ^{59}Fe , ^{62}Cu , ^{64}Cu , ^{67}Cu , ^{67}Ga , ^{68}Ga , ^{75}Sc , ^{77}As , ^{86}Y , ^{89}Sr , ^{89}Zr , ^{94}Tc , $^{99\text{m}}\text{Tc}$, ^{99}Mo , ^{105}Pd , ^{105}Rh , ^{111}Ag , ^{111}In , ^{123}I , ^{124}I , ^{125}I , ^{131}I , ^{142}Pr , ^{143}Pr , ^{149}Pm , ^{149}Tb , ^{153}Sm , $^{154-158}\text{Gd}$, ^{161}Tb , ^{166}Dy , ^{166}Ho , ^{169}Er , ^{175}Lu , ^{177}Lu , ^{186}Re , ^{188}Re , ^{189}Re , ^{194}Ir , ^{198}Au , ^{199}Au , ^{211}At , ^{211}Pb , ^{212}Bi , ^{212}Pb , ^{213}Bi , ^{223}Ra , ^{227}Th , ^{225}Ac , ^3H , ^{11}C , $^{94\text{m}}\text{Tc}$, ^{203}Pb , ^{97}Ru , ^{88}Y , ^{121}Sn . Accordingly, the molecules of the invention are also envisioned as diagnostics for use in assays comprising the detection of cFAP and, in particular, to further discriminate the cFAP from/over other variants of FAP that may exist in a sample, in particular soluble variants as existing in plasma, serum, tissue, blood or body fluids.

Therefore, provided are therapeutic and diagnostic compositions comprising the anti-cFAP antigen binding molecules as disclosed herein, polynucleotides (e.g. in the context of a vector) encoding such molecules, and host cells comprising vectors and/or polynucleotide sequences encoding the molecules. The molecules of the invention are suitable for any immune based

diagnostic assay known in the art and/or described herein (including immunohistochemical assays), in particular, *ex vivo* and *in vitro* diagnostic assays and assay systems as known in the art. For example, methods such as e.g. immunohistochemical staining of biological samples (e.g., comprising tissues or cells) obtained from a patient or measuring the amount of cFAP in
5 a particular tissue can be of value.

The specific and selective anti-cFAP antigen-binding molecules and methods disclosed herein provide for the treatment and diagnosis of diseases characterized by cFAP expression. Accordingly, provided are specific and selective anti-cFAP antigen-binding molecules, antigen
10 binding domains/fragments thereof, polynucleotides encoding such molecules/domains or fragments as well as cells expressing such molecules/domains or fragments for use as a medicament. As is appreciated, cFAP may or may not be expressed by the disease or disease causing cells themselves. A disease also remains characterized by the expression of cFAP where it is not expressed by the diseased cells themselves, but where it is expressed by cells
15 resident within the disease parenchyma, and which are not themselves disease cells. Such cells resident in the disease parenchyma that are not disease cells but that may express cFAP include, but are not limited to CAFs, activated fibroblasts in fibrotic diseases, myofibroblast in scarring, mesenchymal cells in inflammatory diseases.

20 The disease characterized by the expression of cFAP and targeted by the antigen-binding molecules/domains/fragments of the invention or cells expressing such molecules/domains/fragments of the invention may be any disease known in the art such as, but not limited to, cancer, fibrotic cancer, fibrotic diseases (including liver, kidney, and pulmonary fibrosis), inflammatory diseases, scar formation, cardiac diseases (including cardiac
25 fibrosis and heart failure), arthritis, gynecological benign diseases (including endometriosis, fibroids, and Asherman's syndrome), and surgery induced fibrosis. It is preferred that the disease characterized by expression of cFAP and targeted by the antigen-binding molecules/domains/fragments of the invention and methods of their use is endometriosis, fibroids, adenomyosis, atherosclerosis, cFAP+ tumors, cardiac fibrosis, idiopathic pulmonary
30 fibrosis, or surgery induced fibrosis. Accordingly, also provided are methods comprising the administration of a single or multiple dosages of the antigen-binding

molecules/domains/fragments of the invention or pharmaceutical carriers such as VLPs, LNPs, or cells carrying such molecules/domains/fragments of the invention.

The antigen-binding molecules/domains/fragments of the invention and/or compositions comprising such antigen-binding molecules/domains/fragments of the invention are also suitable for use in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. Examples of such immunoassays or immunohistochemical assays are immunoassays or immunohistochemical assays in either a direct or indirect format, and may be single step or multistep (e.g. heterogenous) assays. Examples of such assays are enzyme linked immunosorbent assays (ELISA), enzyme immunoassays (EIA), radioimmunoassays (RIA), Western blot assays, or immunoassays based on detection of luminescence, fluorescence, chemiluminescence or electrochemiluminescence. In certain embodiments, the antigen-binding molecules/domains/fragments of the invention or compositions comprising them can be used in methods for the detection of cFAP. Alternately or additionally, the antigen-binding molecules/domains/fragments of the invention or compositions comprising them can be used in methods to discriminate cFAP from/over soluble variants thereof, including one or more soluble variants as existing in serum, plasma, blood, tissue, or bodily fluids, e.g. spFAP, either *in vivo* or *in vitro*.

The herein described immunoassays (which include immunohistochemical assays) can be performed on any suitable biological sample known in the art and/or described herein. Because the herein described assays are for the specific and selective detection of cFAP, in particular, discriminating over soluble variants thereof, the sample is a biological sample from a subject expected to or demonstrated to contain cFAP. Examples of biological samples suitable for the uses and methods disclosed herein include but are not limited to tissue samples and body fluid samples, e.g. without limitation, plasma, serum and blood.

The term "composition", e.g. where referencing a therapeutic or diagnostic composition as used in accordance with the present invention, relates to a composition which comprises at least one of an anti-cFAP antigen-binding molecule of the invention (or an antigen-binding domain or fragment thereof), a polynucleotide, a vector, and/or a host cell as disclosed herein. It may optionally comprise further molecules capable of altering the characteristics of the

compounds of the invention thereby, for example, stabilizing, modulating and/or enhancing their function. The composition may be in solid or liquid form and may be, inter alia, in the form of (a) powder(s), (a) tablet(s) or (a) solution(s).

- 5 The components of the composition can be packaged in a container or a plurality of containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. A solution for use is prepared by reconstituting the lyophilized compound(s) using either e.g. water-for-injection for therapeutic uses or another desired solvent, e.g. a buffer, for diagnostic purposes. Preservatives and other additives may also be
10 present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. The various components of the composition may be packaged as a kit with instructions for use. Thus, also provided is a kit comprising one or more compositions as disclosed herein.

15 **4. BRIEF DESCRIPTION OF FIGURES**

Figure 1 Comparative ELISA assay of ab1 and ab3 variants fused to a human antibody Fc domain (hFc) to human donor plasma. As a reference, the antibody sibrotuzumab and an antibody construct comprising the Fab domain of 4B9 antibody fused to the hFc ("4B9-hFc") were used. The Fc domain used in 4B9-hFc was the same as
20 used in the ab1 and ab3 Fc fusions. The optical density (OD) measured in the ELISA assay was used to quantify the binding strength of the antibody/antibody constructs to spFAP in human plasma.

25 **Figure 2** Binding activity of antibody constructs to cellular bound FAP (cFAP) via flow cytometry analysis (FCM) of HEK 293F cells transiently transfected with full length human FAP (Uniport-id: Q12884). Mean fluorescence intensity (MFI) was measured and plotted as the difference between human FAP and the isotype control human IgG (MFI). All tested antibody constructs showed binding activity
30 to cFAP.

- Figure 3** Binding activity of antibody constructs to cellular FAP (cFAP) in the presence and absence of undiluted plasma was measured via flow cytometry analysis (FCM) in HEK 293F cells transiently transfected with full length human FAP (Uniport-id: Q12884). Mean fluorescence intensity (MFI) was measured and plotted as the ratio between the binding to cFAP in presence and absence of spFAP in %. Controls were Sibrotuzumab, 4B9 and the isotype control murine IgG.
- Figure 4** Cross-reactivity of antibody constructs to cynomolgus and murine cFAP. The binding affinity of antibodies/antibody constructs against (A) HEK239F transfected with cynomolgus FAP Accession # XP_005573377.1 and (B) HEK293F transfected with murine FAP (Accession # P97321) on cells was measured by flow cytometry. Higher affinity is reflected by higher MFI.
- Figure 5** Improved pharmacokinetic (PK) characteristics of the antibody constructs in mouse plasma ($n \geq 3$). (A) Mean plasma concentration of 4B9-hFc (Pharma) and ab1z12-hFc (single injection of 10mg/kg in BALB/C mice) was monitored over time using human IgG1 ELISA. (B) Summary of PK parameters are summarized in (B).
- Figure 6** BALB/c mice were administered a specific and selective cFAP-mFc construct developed according to the methods of the invention or control molecule formatted as a complete IgG comprising the variable domain of 4B9. Both the specific and selective cFAP antigen binding molecule and 4B9-mFc constructs contained used a murine IgG2a domain as the Fc. The concentration of spFAP in murine plasma was measured by murine FAP sandwich ELISA. spFAP concentration was reduced in mice injected with 4B9-Fc but not reduced in mice injected with the specific and selective cFAP construct.

- Figure 7** Enzymatic activity of (A) recombinant human soluble FAP (Acro Biosystems, Switzerland; FAP-H5244) and (B) spFAP in human plasma was unaffected by the antibody constructs. FAP enzymatic activity was determined using a FAP-substrate-with a quenched dye in the presence of antibody constructs ab1-hFc and ab3-hFc at differing concentrations. Results are presented compared to controls, sibrotuzumab and 4B9-hFc. The measured fluorescence is indicative for the enzymatic activity of FAP. The enzymatic activity of recombinant FAP and of spFAP in human plasma were unaffected by the tested antibody/antibody constructs.
- Figure 8** ADCC reporter assay. The antibody constructs ab1-hFc and ab3-hFc exhibited dose-dependent ADCC activity in different types of fibroblasts: (A) endometrial/uterine (HUF), (B) cardiac (HCF), (C) dermal fibroblasts (Detroit551) and (D) pulmonary (HPF). Sibrotuzumab was used the reference antibody. Relative luminescence was used to quantify the ADCC activity/effector cell activation. Data points represent the mean of technical replicates. A dose-dependent increase in luminescence is observed, substantiating the antibody constructs' efficacy in mediating antibody-dependent cell-mediated cytotoxicity.
- Figure 9** ADCP reporter assay with ab1 and ab3 hFc constructs on HUF and HCF. The antibody constructs ab1-hFc and ab3-hFc exhibited a low dose-dependent ADCP activity in different types of fibroblasts: (A) endometrial/uterine (HUF) and (B) cardiac fibroblasts (HCF). The antibody construct ab1-hFc(DEL) exhibited enhanced ADCP activity. Relative luminescence was used to quantify the ADCP activity/effector cell activation. Data points represent the mean of technical replicates.
- Figure 10** ADCP reporter assay with ab1-hFc constructs comprising afucosylating or DEL Fc modifications on HUF and HPF. Antibody construct ab1-hFc exhibited a relatively low ADCP activity in (A) endometriotic/uterine (HUF) and (B) pulmonary fibroblasts (HPF). ADCP activity was improved by increasing the affinity to FcγR on effector cells by the DEL modification in the Fc region or by afucosylation of the Fc region (ahFc). No nonspecific ADCP activity was observed in the absence of target

cells. Relative luminescence was used to quantify the ADCP activity/effector cell activation. Data points represent the mean of technical replicates.

Figure 11 ADCC reporter assay with ab1-hFc constructs comprising afucosylating (ahFc) or DEL Fc amino acid modifications on HUF, HPF, HCF and Detroit551. Antibody construct ab1-hFc exhibited ADCC activity in (A) endometrial/uterine (HUF), (B) pulmonary (HPF), (C/D) cardiac (HCF) and (E) dermal (Detroit551) fibroblasts. The ADCC activity was improved by increasing the effector function by the DEL modification in the Fc region or by afucosylation of the Fc region. No nonspecific ADCC activity was observed in the absence of target cells. Relative luminescence was used to quantify the ADC activity/effector cell activation. Data points represent the mean of technical replicates.

Figure 12 ADCP assay using PBMC derived macrophages. This assay demonstrates the superior phagocytosis efficacy of ab3z7-ahFc compared to Sibrotuzumab in terms of EC50 and maximal phagocytosis.

Figure 13 Specific uptake of the antibody fluorophore conjugate demonstrating the utility of the antibody in localizing FAP positive cells in complex biology such as cancer or other FAP+ diseases.

Figure 14 Detection of ab3-mFc binding to different tissues. FAP detection based on ab3-mFc staining was low or undetectable across all tested healthy adult tissues.

5. DETAILED DESCRIPTION

5.1 Antigen-binding molecules that selectively and discriminatively bind cFAP

The invention provides antigen-binding molecules comprising or consisting of antibodies, antibody antigen-binding domains and/or antibody antigen-binding fragments that specifically bind to cellular bound fibroblast activation protein (cFAP) as well as polynucleotides encoding and host cells expressing such antigen-binding molecules. The

antigen-binding molecules of the invention are of particular use as therapeutics and reagents for the specific binding and targeting of cFAP, which reagents in particular also discriminate the target antigen, i.e. cFAP, from soluble variants thereof (including the one or more soluble FAP variants as existing in body fluids such as blood, serum, plasma or other tissue fluid. Thus, antibodies and antibody binding fragments of the invention in certain embodiments also discriminate and selectively bind cFAP over non-target binding in the presence of body fluids such as blood, serum, plasma or other tissue fluid.

The antigen-binding molecules disclosed herein specifically and selectively bind cFAP, i.e. cell-membrane bound FAP, known to be a type II transmembrane glycoprotein having the amino acid sequence of SEQ ID NO:1, and do not significantly bind to soluble versions of FAP, known to be one or more variants of FAP having one or more truncated amino acid sequences relative to that of cFAP. An exemplary soluble version of FAP has the amino acid sequence of SEQ ID NO:2. Alternately or additionally the soluble version of FAP is the one or more soluble form known to exist in body fluids such as blood, plasma, serum or other tissue fluid, i.e. spFAP. Additionally, in certain embodiments the antigen-binding molecules do not exhibit significant binding to a body fluid (such as blood, plasma or serum) that does not comprise cFAP.

As demonstrated herein, the specific and selective antigen binding molecules of the invention discriminate cFAP from soluble versions of FAP, including spFAP. Without being bound by any particular explanation, the antigen-binding molecules of the invention recognize and specifically bind to a target epitope not present or not presented in soluble FAP variants in solution. As understood in the art, the target epitope not present or not presented may be an epitope comprising a linear, sequential stretch of amino acids missing in soluble variants of FAP. Alternately or additionally, the target epitope not present or not presented by soluble FAP variants may be a three-dimensional epitope formed from non-sequential residues in the amino-acid sequence brought into spatial proximity by the tertiary structure of cFAP, which three-dimensional epitope is not formed in soluble variants due to changes in amino acid sequence and/or folding. The target epitope not present or not presented by soluble FAP variants may also be unavailable because of steric hindrances that become relevant only in the soluble variants. Thus, the anti-cFAP antigen-binding molecules provided herein may also specifically bind FAP variants other than cFAP provided the target epitope is present.

It is most preferred that the specific and selective anti-cFAP antigen-binding molecules specifically bind to cFAP having the amino acid of SEQ ID NO:1 as expressed on the surface of a cell (e.g. a HEK-293F cell) and discriminate from/over (i.e. do not exhibit specifically binding to) (a) a soluble FAP having the amino acid sequence of SEQ ID NO:2; (b) blood, plasma, serum or other body fluid that does not comprise cFAP; and/or (c) spFAP as known to exist in blood, plasma, serum or other body fluid whether the spFAP is isolated or not.

The anti-cFAP antigen binding molecules provided herein have been exemplified by multiple different working embodiments as disclosed herein (including Fc region containing constructs), which has allowed the determination of consensus V_HH CDR structures that can provide the specific and selective binding to cFAP and provide the discrimination over soluble variants thereof such as soluble FAP having the amino acid sequence of SEQ ID NO:2 and/or the one or more soluble variants of spFAP. However, it is well known in the art that some deviation from the consensus CDR sequences is possible while still retaining the functionality of the specific and discriminative binding exhibited by the exemplary antigen-binding molecules, e.g., as demonstrated at least by standard humanization and affinity maturation protocols. That is, it is known that certain CDR/variable domain residues can be exchanged and sequence variants maintaining the desired functional properties be readily identified using only routine knowledge in the art. Accordingly, the invention provides an antigen-binding molecule comprising or consisting of one or more antibodies, antibody antigen-binding domains or antibody antigen binding fragments that specifically and selectively/discriminatively binds cFAP, which antigen binding molecule comprises or consists of one or more heavy chain variable domains (preferably one or more V_HHs) comprising,

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- (i) a CDR1 having the sequence of X₁X₂TX₃X₄X₅YAX₆G (SEQ ID NO: 3), wherein X₁ is A, G or S; wherein X₂ is P, R or G; wherein X₃ is K, F, R or N; wherein X₄ is S, R or G; wherein X₅ is T, S or N; and wherein X₆ is M, I or L;
- (ii) a CDR2 having the sequence of X₁IX₂WX₃X₄X₅X₆TX₇YX₈DSVKG (SEQ ID NO:4), wherein X₁ is A or V; wherein X₂ is N, W or S; wherein X₃ is S or A; wherein X₄ is G or N; wherein X₅ is T or G; wherein X₆ is I, L, S, T or V; wherein X₇ is S, Q or N; and wherein X₈ is S or T; and/or

30

- (iii) a CDR 3 having the sequence of AADX₁DFRTVGSRPSY (SEQ ID NO:5), wherein X₁ is R, S or K.

The above described anti-cFAP antigen binding molecule may comprise or consist of one or more V_HHs comprising

- (i) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VINWSGTLTSYADSVKG (SEQ ID NO:8) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (ii) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VINWAGTLTSYADSVKG (SEQ ID NO:9) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (iii) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VISWSGTLTSYADSVKG (SEQ ID NO:10) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (iv) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTQYTD SVKG (SEQ ID NO:11) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (v) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTNYTD SVKG (SEQ ID NO:12) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14); or
- (vi) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTNYADSVKG (SEQ ID NO:13) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14).

The above described anti-cFAP antigen binding molecule may also comprise or consist of one or more of the following amino acid sequences

- (i) QVQLVESGGGLVQAGDSLSCVASAPTRSTYAMGWFRQAPGKEREF AAVINWSGTLTSYADSVKGRFTISRDNKNTVFLQMNSLKPDDTAVYYCAADRDFRTVGSRPSYWGQGTPVTVSS ("ab1", SEQ ID NO:15);

- (ii) QVQLVESGGGLVEPGDSLRLSCAASGRTFSSYAIGWFRQAPGKEREFVAAISWSGGTTNYTDSV
KGRFTISRDNKNTVYLQMNSLKPDDEVYCAADRDFRTVGSRPSYWSKGTRVTVSS ("ab3",
SEQ ID NO:16);
- (iii) QVQLVESGGGLVQPGGSLRLSCSASAPTRSTYAMGWFRQAPGKEREFASVISWSGTLTSYADSV
5 KGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGQGTTTVTVSS
("ab1z12", SEQ ID NO:17);
- (iv) QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISWSGGTTQYTDSV
KGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGQGTTTVTVSS
("ab3z7", SEQ ID NO:18);
- 10 (v) QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISWSGGTTNYADSV
KGRFTISRDNKNTLYLQMNSLRPEDTAVYYCAADRDFRTVGSRPSYWGQGTTTVTVSS
("ab3z10", SEQ ID NO:19).

As disclosed above, the CDR, variable domain and V_HH sequences can comprise the recited
15 specific reference sequence(s) or can differ therefrom in one or more amino acid
substitutions. It will be appreciated that amino acid substitutions disclosed above may be
present in none, one, more than one, or all CDRs, variable domains or V_HH sequences of an
antigen binding molecule of the invention. The term "substitution" as used herein refers to
the replacement of an amino acid with another amino acid. The deletion of an amino acid at
20 a certain position and the introduction of one (or more) amino acid(s) at a different position
is explicitly not encompassed by the term "substitution". As described herein above, the
present invention in particular encompasses the recited CDR, variable domain or V_HH
sequences comprising one or more conservative amino acid substitutions.

25 The CDRs, variable domain and V_HH sequences indicated above as comprising one or more
substitutions are referenced herein as "variant" sequences. It is evident that the variant
sequences are functional variants, i.e. having amino acid sequences that may differ from the
reference amino acid sequence but which differing sequence exhibits or maintains the same
functional activity as the reference sequence in the context of the described antigen binding
30 molecules, i.e. imparts or contributes to the specific and selective target antigen binding
activity of the antigen binding molecules of the invention. In this context, "exhibits or
maintains the same functional activity" does not mean that any quantitative parameter of

antigen binding, e.g. the dissociation constant or equilibrium dissociation constant, is the same within experimental error. Rather, a variant sequence that "exhibits or maintains the same functional activity" means that the variant sequence when tested in the same context and under the same conditions as the reference sequence also contributes or imparts the specific and selective anti-cFAP binding as defined herein to the antigen binding molecule. Thus, provided that the antigen binding molecule comprising or consisting of one or more variant sequences maintains the specific and selective anti-cFAP binding as defined herein, it and/or any of its component variant sequences may exhibit an improved, equivalent or decreased quantitative binding parameter relative to the non-variant molecule and/or one or more component sequences, respectively.

It is known in the art that the antibody heavy chain or light chain variable domain (including antibody single chain antigen binding domains such as V_HH) comprises in addition to the above defined 3 CDRs, 4 framework domains ("FWs"). Thus, as well known in the art, the one or more antibodies, antibody antigen binding domains or antibody antigen binding fragments (including, e.g. one or more CDRs, variable domains or V_HH sequences) of the antigen binding molecules of the invention comprise in addition to the above-defined CDRs, the necessary framework domains. The sequences of the necessary/surrounding FWs can be chosen by the skilled person using standard methods routinely practiced in the art. It is appreciated that the skilled person will choose the appropriate sequences for the FWs such that the resultant component one or more antibodies, antibody antigen binding domains or antibody antigen binding fragments (comprising at least one CDR, variable domain or V_HH sequence as defined herein) of the antigen binding molecule imparts or contributes to the specific binding and selective anti-cFAP binding activity of the molecule.

In particular embodiments, the anti-cFAP antigen binding molecule of the invention comprises one or more of an amino acid sequence having at least 85%, at least 90%, or at least 95% sequence identity to any of SEQ ID NO:15, 16, 17, 18, and 19. As defined herein, amino acid sequences having such sequence identity to any of SEQ ID NO:15, 16, 17, 18 and 19 are variant amino acid sequences, i.e. functional variants as defined herein above. The total number of substitutions in the variant sequences of SEQ ID NO:15, 16, 17, 18, and 19 may be at most 18, at most 17, at most 16, at most 15, at most 14, at most 13, at most 12, at most 11, at most 10,

at most 9, at most 8, at most 7, at most 6, at most 5, at most 4, at most 3, at most 2, or the variant sequence may comprise only 1 substitution relative to the reference sequence. The one or more substitutions may be in one or more CDR, one or more FW, or at least one CDR and at least one FR.

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As used herein, the term “% sequence identity” in connection with amino acid sequences of polypeptides/peptides and/or nucleic acid sequences or nucleic acid molecules describes the number of matches of identical amino acid or nucleic acid residues of two or more aligned sequences as compared to the number of residues making up the overall length of the compared sequences (or the overall compared portions thereof). Using an alignment of two or more sequences or subsequences, the percentage of residues that are the same may be determined when the (sub)sequences are compared and aligned for maximum correspondence over a window of comparison, or over a designated region as measured using a sequence comparison algorithm as known in the art, or when manually aligned and visually inspected. Non-limiting examples of algorithms for use in determining sequence identity include, for example, those based on the NCBI BLAST algorithm (Altschul et al., Nucleic Acids Res. 25(1997), 3389-3402), CLUSTALW computer program (Thompson, Nucl. Acids Res. 2(1994), 4673-4680) or FASTA (Pearson and Lipman, Proc. Natl. Acad. Sci., 85(1988), 2444). Although the FASTA algorithm typically does not consider internal non-matching deletions or additions in sequences, i.e. gaps, in its calculation, this can be corrected manually to avoid an overestimation of the % sequence identity. CLUSTALW, however, does take sequence gaps into account in its identity calculations. Also available are the BLAST and BLAST 2.0 algorithms (Altschul et al., Nucl Acids Res., 25(1997), 3389).

25 The invention also provides a second anti-cFAP antigen binding molecule that binds to the same epitope of cFAP as (a) a V_HH comprising or consisting of the amino acid sequence of SEQ ID NO:15, 16, 17, 18 or 19; or (b) a first antigen binding molecule comprising or consisting of a V_HH having the amino acid sequence of SEQ ID NO:15, 16, 17, 18 or 19. The specific epitope of cFAP or partial sequence thereof bound by any of the immediately above defined V_HH domains or an antigen binding molecule comprising an above-defined V_HH domains, can be identified by any suitable epitope mapping method known in the art. Examples of such methods include for example, mass spectrometric analysis of the antigen binding molecules

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of the invention bound to cFAP peptides of various lengths. In another example, NMR spectroscopy or X-ray crystallography can be used to identify the epitope bound by an antigen-binding molecule of the present invention. Once identified, the epitopic fragment which binds an antigen binding molecule of the present invention can be used as an immunogen to obtain
5 additional antibodies binding the same epitope.

5.2 Production and Engineering of antigen binding molecules

As defined herein the antigen binding molecules of the invention can comprise one or more
10 antibodies, antibody antigen binding domains or antibody antigen binding fragments (including, e.g. one or more CDRs, variable domains or V_HH sequences), and may further comprise an antibody Fc region or an Fc-receptor binding domain. Unless otherwise specified, the terms “antibody”, “antibodies”, and analogous terms relate to full immunoglobulin molecules and encompass naturally-occurring forms of antibodies (including but not limited
15 to IgG, IgA, IgM, IgE) as well as recombinant antibody constructs including but not limited to single-chain antibodies, chimeric antibodies, humanized antibodies, antibody-fusion proteins, and multi-specific antibodies; as well as antigen binding fragments and derivatives of all of the foregoing. As known in the art, antibodies comprise a variable region (known in the art as an “Fv region”, and/or an “Fv domain”) that is formed from paired variable domains from both
20 the heavy and light chains, which variable domains and/or the Fv domain interact with the antigen. The term Fv region does not include constant regions of the heavy and/or light chains.

The terms “antibody”, “antibodies”, and analogous terms as used herein also refer to the antigen binding fragment thereof, which may be referenced herein as antibody antigen
25 binding domain, antibody antigen binding fragment, and/or, simply antigen binding fragment. These terms refer to one or more domains or fragments of an antibody that retain the ability to specifically bind to the target antigen, e.g., cFAP, as known in the art, including but not limited to antigen binding fragments comprising an Fv domain, i.e., paired heavy and light chain variable domains, such as Fab, Fab', F(ab')₂, and Fv fragments as well as recombinant
30 constructs such as single-chain Fv domains, known in the art as scFvs. The terms also includes antibody antigen binding fragments that comprise a single, unpaired heavy or light chain variable domain as known in the art that retains the ability to specifically and selectively bind

antigen as defined herein, including but not limited to single domain antibodies (also referenced in the art as sdAbs, dAbs, and/or nanobodies) and V_HH domains based on the heavy chains of camelids. It is preferred that the antigen binding molecule of the invention comprises one or more antibody, which antibody is a V_HH.

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The antibodies and antigen binding domains or fragments (including, e.g. one or more CDRs, variable domains or V_HH sequences) may be polyclonal or monoclonal, preferably monoclonal. The terms “monoclonal”, “monoclonal composition”, and analogous terms as used herein with reference to an antigen binding molecule, or a component antibody, antibody antigen binding domain, or antibody antigen binding fragment, refer to a population of antibody polypeptides or fragments thereof produced from a single B cell clone, which population contains only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen. This is in contrast with “polyclonal” antibodies and compositions, which term(s) refer to a population of antibody polypeptides or antigen binding fragments that contain multiple species of antigen binding sites. Also included are modified forms of any antigen binding molecule or component antibody, antibody antigen binding domain or antibody antigen binding fragment such as humanized or chimeric versions thereof, as well as recombinant constructs, such as fusion proteins, wherein the antigen binding molecule further comprises, e.g. an Fc region or an Fc receptor binding domain. Non-limiting examples of antigen binding molecules of the invention include an antibody antigen binding domain or fragment (including comprising or consisting of one or more CDRs, variable domains or V_HH sequences) recombinantly fused to an antibody Fc region or Fc receptor binding domain. The antigen binding molecule may also comprise additional domain(s), e.g. for the isolation and/or preparation of recombinantly produced molecules as known in the art.

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The antigen binding molecules of the invention and/or components thereof (including without limitation, antibodies, antibody antigen binding domains or fragments, CDRs, variable domains, single domain antigen binders (e.g. a V_HH), Fc regions and Fc receptor binding domains) may be prepared by a variety of techniques routinely used in the art. For example, immunizing a non-human animal with one or more antigens of interest (i.e. cFAP, which may be in one or more formats and/or derived from one or more species) and subsequently

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isolating antigen-reactive, antibody producing B-cells. It is further preferred that the positive clones are further subjected to a negative selection, excluding clones that specifically or significantly react with soluble versions of FAP, including but not limited to FAP having the amino acid sequence of SEQ ID NO:2 and/or spFAP. Alternately or additionally, the negative selection can exclude clones that significantly react with body fluids such as blood, serum or plasma known or believed to contain spFAP (whether or not spFAP has been detected in the specific body fluid used). Methods of isolating and/or selecting clones (positively or negatively) that produce antibodies having desired characteristics are well known in the art. As known in the art, the peptide immunogen of the target antigen may be coupled to adjuvant-carrier, e.g. keyhole limpet hemocyanin (KLH), and/or administered together with an adjuvant composition, e.g. Freund's complete or incomplete adjuvant, to improve immunogenicity. Animals can be immunized according to a standard schedule, such as weekly, monthly or a combination of weekly and monthly, depending on the animal, antigen and titer of antibody developed. To determine the response of the animals, antibody titer in serum can be tested according to standard procedures. The peripheral blood mononuclear cell (PBMC) fraction of positive animals can be isolated and antigen-reactive B cells purified using standard techniques, such as ELISA or column based techniques to purify reactive B-cells from serum as described in, e.g. Seeber et al., PLoS One. 9(2014), e86184. As noted, such screening methods preferably comprise a negative selection step to identify and exclude clones exhibiting cross-reactivity with soluble versions of FAP, spFAP and/or body fluids known or believed to contain spFAP. Selected can then be used for subsequent recombinant processing.

Another suitable method for producing or isolating antibodies and antibody antigen binding fragments of the invention include, but are not limited to, methods that select a recombinant antibody or antibody antigen binding domain or fragment (including a single domain binder such as a V_HH) from a peptide or protein library (e.g., but not limited to, a bacteriophage, bacterial, mammalian cell, ribosome, oligonucleotide, RNA, cDNA, or yeast display library) using binding activities of interest. For example, as exemplified in the working examples, antigen binding molecules or components thereof can be selected from such libraries by positively selecting for specific binding to cFAP (e.g. as expressed on the surface of a cell either endogenously or recombinantly) and negatively selecting for binding to soluble FAP, spFAP and/or a body fluid such as blood, plasma or serum. Display libraries are well known in the art

and are, for example, available from various commercial vendors including but not limited to Cambridge Antibody Technologies (Cambridgeshire, UK), MorphoSys (Martinsried/Planegg, Del.), Biovation (Aberdeen, Scotland, UK) and Bioinvent (Lund, Sweden). Again, selected clones can be processed according to routine methods for subsequent recombinant
5 processing into antigen binding molecules of the invention.

Accordingly, the present invention also provides nucleic acid molecules encoding the antigen-binding molecules disclosed herein as well as antigen-binding components thereof, preferably, encoding a specific and selective anti-cFAP V_HH. As used herein, "nucleic acid
10 molecule", "nucleic acid sequence", "polynucleotide" and analogous terms include both genomic DNA and cDNA, as well as RNA capable of driving expression of a peptide or polypeptide antigen binding molecule of the invention. It is understood that the term "RNA" as used herein comprises all forms of RNA including mRNA, tRNA and rRNA but also genomic RNA, such as in case of RNA of RNA viruses. Preferably, embodiments reciting "RNA" are
15 directed to mRNA. The nucleic acid molecules/nucleic acid sequences of the invention may be of natural as well as of synthetic or semi-synthetic origin. Thus, the nucleic acid molecules may, for example, be nucleic acid molecules that have been synthesized according to conventional protocols of organic chemistry, according to recombinant methods, or produced semi-synthetically, e.g. by combining chemical synthesis and recombinant methods. The
20 person skilled in the art is familiar with the preparation and the use of such nucleic acid molecules.

The particular embodiments, the invention in particular provides a polynucleotide encoding a polypeptide comprising or consisting of any of SEQ ID NOs:3 to 19, preferably any of SEQ ID
25 NO:15, 16, 17, 18 or 19, or a variant thereof as defined herein above.

Also provided are vectors comprising the nucleic acid molecules encoding the antigen binding molecules of the invention or one or more components thereof. As used herein, the term "vector" relates to a circular or linear nucleic acid molecule that can autonomously replicate
30 in a host cell into which it has been introduced. Non-limiting examples of vectors suitable for use in the present invention include cosmids, plasmids (e.g. naked or contained in liposomes), viruses (e.g. lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) and

bacteriophages. However, the art provides many suitable vectors, the choice of which depends on the desired function. The development and use of suitable vectors is well documented in the art; see, for example, the techniques described in Sambrook and Russel "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory, N.Y. (2001) and Ausubel, "Current Protocols in Molecular Biology", Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Vectors of use in connection with the present invention comprise a nucleic acid sequence encoding the anti-cFAP antigen binding molecules and/or one or more component domains/regions thereof as disclosed herein. As such, the vectors of use in connection with the present invention may encode a peptide or polypeptide comprising or consisting of any of SEQ ID NOs: 3 to 19, preferably any of SEQ ID NOs: 15 to 19.

With regard to the term "vector comprising" as used herein, it is understood in the art that further nucleic acid sequences are present in the vectors that are necessary and/or sufficient for desired vector activity in the host cell, e.g. drive replication of the vector (and, thus the encoding nucleic acid sequences) and/or to direct the host cell express the antigen binding molecule of the invention or a component domain thereof. Such further nucleic acid sequences include but are not limited to sequences controlling vector replication and/or expression of a desired sequence in the particular cell system. For example, the vectors may comprise the nucleic acid molecule encoding a desired peptide or polypeptide sequence operably linked and/or under the control of regulatory sequences. The term "regulatory sequence" refers to DNA sequences that are necessary to effect the expression of coding sequences to which they are operably linked. The term "control sequence" is intended to include, at a minimum, all components the presence of which may also be necessary for expression, and may further include additional advantageous components, e.g. to allow replication. As is understood in the art, the nature of such regulatory and control sequences differs depending upon the host organism. For example, in prokaryotes, control sequences generally include promoters, ribosomal binding sites, and terminators. In eukaryotes control sequences generally include promoters, terminators and, in some instances, enhancers, transactivators and/or transcription factors.

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The vectors of use in the present invention are preferably expression vectors. An expression vector is capable of directing the replication and the expression of the nucleic acid molecule

of the invention in a host cell and, accordingly, provides for the expression of an anti-cFAP antigen binding molecule or a component domain thereof as disclosed herein. Suitable expression vectors have been widely described in the literature and the determination of the appropriate expression vector for a particular cell system can be readily made by the skilled person using routine methods. Preferably, the vectors disclosed herein comprise a recombinant polynucleotide (i.e. a nucleic acid sequence encoding the anti-cFAP antigen binding molecule or component domain thereof) as well as expression operably linked control sequences. The vectors as provided herein may further comprise a promoter. The herein described vectors may also comprise a selection marker gene and a replication-origin ensuring replication in the host. Moreover, the herein provided vectors may also comprise a termination signal for transcription. Expression vectors as known in the art may drive transient or constitutive expression in a host cell.

The nucleic acid molecules and/or vectors of the invention can be designed for transfection into prokaryotic or eukaryotic host cells by any means known in the art or described herein. Non-limiting examples of suitable methods include chemical based methods (polyethylenimine, calcium phosphate, liposomes, DEAE-dextrane, nucleofection), nonchemical methods (electroporation, sonoporation, optical transfection, gene electrotransfer, hydrodynamic delivery or naturally occurring transformation upon contacting cells with the nucleic acid molecule of the invention), particle-based methods (gene gun, magnetofection, impalefection) phage vector-based methods and viral methods. For example, expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, Semliki Forest Virus or bovine papilloma virus, may be used for transfection of the nucleic acid molecules into targeted cell population. Additionally, baculoviral systems can also be used as vector in eukaryotic expression system for the nucleic acid molecules of the invention.

The term "prokaryote" is meant to include all bacteria which can be transformed, transduced or transfected with DNA or DNA or RNA molecules for the expression of a protein of the invention. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, *E. coli*, *S. typhimurium*, *Serratia marcescens*, *Corynebacterium* (glutamicum), *Pseudomonas* (fluorescens), *Lactobacillus*, *Streptomyces*, *Salmonella* and *Bacillus subtilis*. The

term "eukaryotic" is meant to include yeast, higher plant, insect and mammalian cells. Non-limiting examples of mammalian host cells typically used in the art include, Hela, HEK293 (including HEK-293F), H9, Per.C6 and Jurkat cells, mouse NIH3T3, NS/O, SP2/O and C127 cells, COS cells, e.g. COS 1 or COS 7, CV1, quail QC1-3 cells, mouse L cells, mouse sarcoma cells, Bowes melanoma cells and Chinese hamster ovary (CHO) cells.

When recombinant expression vectors encoding the antigen binding molecules or a component domain thereof are introduced into host cells, the molecules or component domains are produced by culturing the host cells for a period of time sufficient to allow for expression of the molecules and/or domains in the host cell or, preferably, to allow for secretion of the molecule and/or domain into the culture medium in which the host cells are grown. The molecules of interest can be recovered from the culture medium using standard protein purification methods. The invention further provides an antigen binding molecule obtainable by any of the methods disclosed herein.

It will be appreciated that variations on the above procedures are within the scope of the present invention. For example, recombinant DNA technology may be used to remove or modify the DNA sequences encoding the antigen binding molecules or component domains thereof disclosed herein, e.g. V_HH domains as defined herein above. For example, recombinant DNA technology may be used to remove parts of the encoding sequence(s) that are not necessary for maintaining specific and selective binding to the antigen(s) of interest. The molecules expressed from such truncated DNA molecules are also encompassed by the invention. Additionally, also provided are bifunctional antigen binding molecules, e.g. comprising one or more antibodies, antibody antigen binding domains or antibody antigen binding fragments (including, e.g. one or more CDRs, variable domains or V_HH sequences as defined herein) providing specificity to cFAP and an antibody or antibody antigen binding domain or fragment specific for an antigen other than cFAP.

The antigen binding molecules or component domains thereof as defined herein can comprise or consist of derivatives of antibodies or one or more domains thereof, produced, for example, by adding exogenous sequences to modify immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable

characteristic. Generally, with respect to the antigen binding domains part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the FW and/or constant regions are replaced with human or other amino acids.

5 Also provided are humanized versions of the antigen binding molecules disclosed herein, i.e. comprising the CDRs and V_HH sequences as disclosed herein above. As well known in the art, "humanization" (to produce a humanized version of a parent antibody) refers to recombinantly engineering an antibody antigen binding domain using CDRs derived from a non-human donor immunoglobulin in the context of human derived FWs and, optionally,
10 constant domains. During the engineering, FW and/or CDR residues may be altered to preserve binding affinity and activity, e.g. specific and selective binding to cFAP. Methods to humanize antibodies and antibody domains are well known in the art, e.g. as disclosed in Queen et al., Proc. Natl. Acad Sci USA 86(1989), 10029-10032; Hodgson et al., Bio/Technology 9(1991) 421.

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5.3 Characterization of binding activity

The antigen binding molecules of the invention exhibit specific binding to cFAP and also discriminate from soluble variants of FAP, such as soluble FAP having the amino acid sequence
20 of SEQ ID NO:2 and the one or more soluble FAP variants present in blood, serum or plasma (spFAP). Accordingly, the antigen binding molecules of the invention also do not exhibit non-specific binding to body fluids known or believed to contain spFAP (such as, in non-limiting examples, blood, serum and plasma). The antigen binding molecules of the invention comprise one or more antibody antigen binding domains or fragments as defined herein. Thus, as used
25 herein, the phrase "specifically binds" in the context of an antigen binding molecule reacting with cFAP indicates that the cFAP is bound to the antigen binding molecule via an antigen-antibody reaction. In particular embodiments, the reaction is a V_HH-antigen reaction. As is defined herein above, the term discriminates from/over indicates that the antigen binding molecules bind to the target antigen (i.e. cFAP), but do not specifically bind to soluble variants
30 of FAP and/or do not exhibit significant binding to or other reaction with body fluids known or believed to contain one or more soluble FAP variants (spFAP) such as blood, serum, plasma, or other tissue fluid.

The specific and selective binding to cFAP over one or more soluble FAP variants, including spFAP and/or body fluids known or believed to contain spFAP, may be assessed by any method known in the art or as described herein, in particular by comparing binding activity for cFAP to that for one or more soluble variants of FAP. Because cFAP is cellularly bound FAP, it is preferred that the binding activity of an antigen binding molecule of the invention to cFAP is assessed by the determining the binding to cFAP as expressed on the surface of a cell. In this regard it is appreciated that assay protocols for determining binding to cFAP will not be identical to those used to determine binding to soluble variants of FAP, e.g. at least because the ligand soluble FAP can be used in solution or in an assay format not applicable to cells or the cell membrane bound cFAP. Nevertheless, the modification of assay parameters to allow comparison between the binding activity to cFAP and one or more soluble FAP variants can be readily made by the skilled person using standard procedures in the field and or as described herein. It is as used herein the term discriminates from/over indicated that the binding activity to cFAP is at least 20 fold greater than that to one or more soluble variants (e.g. spFAP), i.e. is at least one order of magnitude greater than that to one or more soluble variants of spFAP.

It is preferred that the binding activity determinative of specific and selective binding to cFAP is the EC_{50} for binding to cFAP, in particular relative to one or more soluble variants of FAP. It is further preferred that the assays to determine the EC_{50} values for binding to cFAP and/or one or more soluble variants of FAP are calibrated by the use of control FAP binding molecules, i.e. calibrated to provide determined results on testing the control molecules, preferably sibrotuzumab and/or 4B9.

With respect to the determination of EC_{50} for binding to the soluble variant(s) of FAP, spFAP, the assay conditions may be calibrated/selected so that the EC_{50} of sibrotuzumab or 4B9 binding to the spFAP is less than 1 nM or less than 10 nM, respectively. As explained herein, spFAP is also known to exist in body fluids such as blood, plasma and serum. Thus, the determination of the EC_{50} value for binding to spFAP need not necessarily be performed on spFAP or an isolated soluble FAP variant, but can be assessed against a body fluid known to contain spFAP. Where a body fluid is used, it is preferred that it is undiluted human plasma containing spFAP at a concentration of at least 80 ng/mL. Therefore, in certain embodiments,

the determination of EC_{50} for binding to the soluble variant(s) of FAP is the determination of the determination of EC_{50} for binding to undiluted human plasma containing spFAP at a concentration of at least 80 ng/mL, wherein the assay conditions are calibrated/selected so that the EC_{50} of sibrotuzumab or 4B9 binding to the undiluted human plasma is less than 1 nM or less than 10 nM, respectively.

With respect to the determination of EC_{50} for binding to cFAP, an exemplary assay is the use of flow cytometry. It is preferred that flow cytometry assay conditions are calibrated/set so that EC_{50} value for binding to cFAP is less than 5 nM. It is further preferred that under the calibrated assay conditions (a) the EC_{50} of binding to said cFAP is within at least 1-fold of the EC_{50} of sibrotuzumab or 4B9 as assessed under the same conditions; and/or (b) the maximum binding to said cFAP is within 1-fold of the maximum binding of sibrotuzumab or 4B9 as assessed under the same conditions. The cFAP may be expressed by any suitable cell known in the art or described herein, e.g. as suitable for the flow cytometry assay. In a particular embodiment, the cFAP has the amino acid of SEQ ID NO:1 and the cell used to express said cFAP is a HEK-293F cell. The specific and selective binding to cFAP by antigen-binding molecules of the invention can also be identified by the antigen binding molecules exhibiting one or both of (a) an apparent K_d of binding to cFAP of less than 2nM or (b) an EC_{50} of binding to cFAP of less than 2 nM, wherein the assay conditions are calibrated/set so that (c) the EC_{50} of binding to said cFAP is within at least 1-fold of the EC_{50} of sibrotuzumab or 4B9 assessed under the same conditions; and/or (d) the maximum binding to cFAP is within 1-fold of that of sibrotuzumab or 4B9 as assessed under the same conditions.

Where the assays for binding to cFAP and the one or more variant(s) of soluble FAP, e.g. spFAP and/or a body fluid known or believed to contain spFAP, are calibrated/set as outlined herein above, specific and selective binding is determined where the EC_{50} value for binding to cFAP is less than 5 nM and the EC_{50} binding to one or more soluble variants of FAP is at least 20-fold weaker. Alternately or additionally, the specific and selective binding may also be identified using assays calibrated/set as outlined above by determining and comparing the TOP (MFI) of the antigen-binding molecule to cFAP relative to that of one or more soluble variants of FAP, such as recombinantly expressed on the surface of HEK 293F cells, and (ii) the EC_{50} of said molecule for binding to one or more soluble FAP, e.g. spFAP. In a non-limiting example, the

anti-cFAP antigen-binding molecule of the invention is specific and selective for cFAP (over sFAP) where

- (i) the EC₅₀ or TOP (MFI) for binding to cFAP is at least 60% of the EC₅₀ or TOP (MFI) of sibrotuzumab when assessed under the same conditions; and
- 5 (ii) the EC₅₀ of said molecule for binding to one or more soluble variants of FAP is
 - (a) weaker than 40nM,
 - (b) not detectable, or
 - (c) incalculablewhen assessed in an assay calibrated/having conditions set such that the EC₅₀ for
- 10 sibrotuzumab or 4B9 binding to said one or more soluble variants of FAP is below 1nM or below 10nM, respectively.

In a particular embodiment, the anti-cFAP antigen-binding molecule of the invention is specific and selective for cFAP (over sFAP) where

- (i) the EC₅₀ or TOP (MFI) for binding to cFAP having the amino acid sequence of SEQ ID NO
- 15 1 and expressed on the surface of a HEK-293F cell is at least 60% of the EC₅₀ or TOP (MFI) of sibrotuzumab for binding to said cFAP when assessed under the same conditions; and
- (ii) the EC₅₀ of said molecule for binding to undiluted human plasma having an spFAP concentration of at least 80 ng/mL is
 - (a) weaker than 40nM,
 - 20 (b) not detectable, or
 - (c) incalculablewhen assessed in an assay calibrated/having conditions set such that the EC₅₀ for
- sibrotuzumab or 4B9 binding to said undiluted human plasma is below 1nM or below
- 25 10nM, respectively.

In a nonlimiting example, the cells expressing the cFAP, e.g. of SEQ ID NO:1, for use in the determination of EC₅₀ and/or MFI express an average of approximately 30000 copies of cFAP molecules per cell. The cells can be exposed to a dilution series of the antigen-binding molecules to be tested and the bound molecules detected according to standard protocols as

30 known in the art or as described herein. A sigmoidal dose-response curve (e.g. a four-parameter log model) can be used to fit the data and the EC₅₀ determined as the concentration at which the fitted curve reaches 50% of the maximum response, also as is well known in the

art. In a particular embodiment, the dilution series comprises incubating 100,000 cFAP expressing cells with antigen binding molecule concentrations of at least 25, 5, 1, 0.2, 0.04, 0.008, 0.0016, and 0.00032 nM. The medium comprising the antigen binding molecules to be tested can be any suitable medium such as, but not limited to PBS supplemented with 1% BSA

5 or undiluted human plasma, wherein the antigen binding molecules incubated in the medium for 1 hour at room temperature. To aid detection or isolation, the antigen binding molecules can optionally comprise known purification/isolation/detection tags or domains such as, but not limited to, antibody Fc domains, e.g. a murine IgG2a (SEQ ID NO: 20). The antigen binding molecules can be incubated with the cells for 30 minutes at 4°C and, following standard wash

10 protocols, detected by any suitable method. For example, where the antigen binding molecules comprise an antibody Fc domain, a suitable secondary labelled anti-Fc antibody. Where the antigen binding molecules comprise a murine IgG2a, a suitable secondary antibody can be an anti-mIgG2a-PE. Quantification of binding can also be performed according to standard protocols, e.g. measuring the Mean Fluorescence Intensity (MFI) by flow cytometry.

15 Appropriate controls may include unstained or isotype control, e.g. to allow for correction of non-specific signal or autofluorescence, and/or Fluorescence Minus One (FMO) controls for each fluorochrome. The MFI values can be normalized by subtracting the isotype MFI from treated samples.

20 In a further non-limiting example, the specific and selective binding activity of the anti-cFAP antigen-binding molecule of the invention can be determined in a competitive binding experiment such as the detection and comparison of binding activity for cFAP in the presence or absence of one or more soluble variants of FAP, e.g. spFAP and/or a body fluid known or believed to contain spFAP (e.g. without limitation plasma or serum, preferably human plasma

25 or human serum). In a non-limiting example, the anti-cFAP antigen-binding molecule of the invention is specific and selective for cFAP (over spFAP) where the MFI in a flowcytometry assay comprising the use of cFAP (e.g. having the amino acid sequence of SEQ ID NO:1 and recombinantly expressed on the surface of HEK 293F cells) in the absence of one or more soluble variants of FAP (e.g. in the absence of spFAP or in the absence of human plasma

30 containing FAP) decreases by less than 40%, less than 30%, less than 20%, less than 15% or, preferably less than 12% at concentrations below 1 nM as compared to the MFI as determined in the same assay under the same conditions but in the presence of one or more soluble

variants of FAP (e.g. in the presence of spFAP or, preferably, in the presence of human plasma containing FAP). It is preferred that the assay is calibrated/the conditions set so that the MFI as determined for sibrotuzumab or 4B9 in the same assay and under the same conditions decreases by at least, 50%, at least 60%, at least 70%, at least 80%, or preferably at least 90% in the presence of one or more soluble variants of FAP (e.g. in the presence of spFAP or in the presence of human plasma containing FAP) at concentrations below 1 nM as compared to the MFI as determined under the same conditions in the absence of the one or more soluble variants of FAP (e.g. in the absence of spFAP or, preferably, in the absence of human plasma containing FAP). The antigen binding molecule of the invention can also be specific and selective for cFAP over one or more soluble variants of FAP, e.g. human plasma, where the ratio of binding to cFAP in the presence of undiluted human plasma known or believed to contain spFAP, (e.g. at concentration of at least 80ng/mL) relative to the binding in the absence of said undiluted human plasma is at least 80% at an antigen-binding molecule concentration of 0.8nM, at least 60% at an antigen-binding molecule concentration of 0.16 nM, or at least 50% at an antigen-binding molecule concentration of 0.0032 nM. It is preferred that the comparative assay in this context exhibits under the same conditions a ratio of binding for 4B9 or sibrotuzumab to cFAP in the presence of said undiluted human plasma relative to the binding in the absence of said undiluted human plasma of less than 10% or less than 25%, respectively, wherein the 4B9 or sibrotuzumab concentration is less than or equal to 0.8nM and greater than or equal to 0.0032 nM, e.g. 0.8 nM, 0.016 nM, or 0.0032 nM.

Similar to the competitive binding assays outlined above, the specific and selective binding activity of the anti-cFAP antigen-binding molecule of the invention can be determined by determining and comparing any other activity of the antigen binding molecule in the presence or absence of one or more soluble variants of FAP, e.g. spFAP and/or a body fluid known or believed to contain spFAP (e.g. without limitation plasma or serum, preferably human plasma or human serum). The specific and selective activity can be determined where the measured activity is unaffected (within experimental error) by the presence of one or more soluble variants of FAP, and, in particular, remains unaffected by increasing concentrations of one or more variants of soluble FAP, e.g. up to a concentration of 100ng/ml, 200 ng/ml, 500 ng/ml or 1000 ng/ml. As control, a decrease in activity of the known FAP binding molecules sibrotuzumab or 4B9 will be observed with increasing concentration of soluble FAP in the

same assay under the same conditions. The cytotoxic activity of an antigen binding molecule towards cFAP expressing cells can be assessed in the presence and absence of one or more soluble variants of FAP according to any method known in the art or described herein. In a non-limiting exemplary assay, the cytotoxic activity can be assessed using a bispecific antigen
5 binding molecule of the invention, e.g. that cross-links cFAP and an antigen on cytotoxicity mediating immune cell such as CD3 on a T cell, which assay further comprises T cells, again, as known in the art. The resistance to soluble variants of FAP as described herein can be assessed by incubating the antigen binding molecules of the invention in the presence and absence of one or more soluble variants of FAP, e.g. spFAP and/or in serial dilution (and/or equivalent
10 increasing concentrations of the soluble FAP) prior to use in the assay and comparing the alteration in cytotoxic activity.

As set forth above, the present invention resolves many of the disadvantages of anti-FAP antigen binding molecules of the art, in particular by specific and selective binding to cFAP.
15 Known anti-FAP binding molecules such as sibrotuzumab and 4B9 bind soluble FAP and, thus, cannot significantly discriminate cFAP from any soluble variant(s) that may also be present in the target sample or tissue. Thus, the soluble variant(s) of FAP (frequently present in high concentrations in body fluids such as blood, serum or plasma) may block or act as a sink for therapeutics targeting cFAP, preventing cFAP expressing cell recognition and/or requiring
20 prohibitive doses of anti-cFAP agents to achieve effect. In the present invention, it has been found that it is possible to generate antigen binding molecules that specifically and selectively bind cFAP even in the presence of high concentrations of soluble variants of FAP. Because the antigen binding molecules of the invention do not significantly bind soluble FAP, their activity is not significantly influenced by the presence of soluble FAP, e.g. spFAP at levels found in
25 blood serum or plasma *in vivo*. In the context of cytotoxic antigen binding molecules as described herein, the invention thus provides antigen binding molecules having cytotoxic activity that is not affected or resistant to the presence of soluble variants of FAP, e.g. spFAP, e.g. at levels of greater than or equal to 40 ng/mL, 100 ng/mL, or a physiologically relevant concentration.

30

As provided herein, the present invention provides anti-cFAP antigen binding molecules particularly suited for the treatment of diseases characterized by the presence of cFAP. In

certain embodiments, the anti-cFAP antigen binding molecules are suited for treatment in subjects having a concentration of soluble FAP (which may comprise one or more soluble FAP variants) of at least 40 ng/mL.

5 In view of the selectivity of the anti-cFAP antigen binding molecules disclosed herein, the molecules and compositions of the invention are referenced as not being affected or as being resistant to one or more soluble FAP variants, e.g. spFAP. The term "resistant to soluble FAP", "resistant to one or more soluble FAP variants" (and related terms) as used herein refers to the fact that the activity of the anti-cFAP antigen binding molecule, e.g. binding activity or
10 cytotoxic activity, is not affected by various physiological concentrations of spFAP.

5.4 Diagnostic and pharmaceutical compositions

As described herein above, the anti-cFAP antigen binding molecules discriminate, over soluble
15 forms/variants of FAP. Thus, the antigen binding molecules are in particular suitable for the specific targeting and detection of cFAP that is unaffected by the presence of soluble variants of FAP as known to be present in body fluids such as blood, serum and plasma.

Accordingly, the present invention further relates to compositions, e.g. diagnostic or
20 pharmaceutical compositions, comprising at least one of (i) an antigen binding molecule of the invention, (ii) a nucleic acid molecule of the invention, (iii) a vector of the invention, (iv) a host cell of the invention, and/or (v) an antigen binding molecule produced or obtained by a method of the invention.

25 5.4.1 Diagnostic compositions

As noted, the anti-cFAP antigen binding molecules provided herein are of use in the detection of cFAP, in particular, relative to and thus discriminating over soluble variants of FAP that may be present in a sample. In the most preferred embodiments, the anti-cFAP antigen binding
30 molecules of the invention are of use in the detection of cFAP in the presence of body fluids known, suspected or believed to contain one or more soluble variants of FAP. The skilled person is well aware of how to determine whether a sample contains cFAP using an antigen binding molecule of the invention. Non-limiting examples of suitable methods include *in vivo*

assays wherein the antigen binding molecule is conjugated to suitable detectable reagent or moiety such as a radionuclide, or contrast agent (e.g. for MRI or CT), and *in vitro* assays such as immunohistochemical and immunocytochemical methods, Western blotting, ELISA, and immunoassays based on detection of absorbance, luminescence, fluorescence, chemiluminescence or electrochemiluminescence. The diagnostic methods may include the use of suitable controls to ensure that any positive or negative result is reliable. Suitable positive as well as negative controls can be designed and included in the experimental setup by a skilled person using conventional methods and the teaching of the present disclosure.

10 The biological samples wherein the cFAP is detected in accordance with the methods disclosed herein include subject-derived samples or preparations. The subject-derived sample may be any sample known, determined or suspected of comprising cFAP and include, but are not limited to subject tissue samples that may further comprise or be contaminated with blood, plasma, serum or other body fluids. The methods also encompass the analysis of subject-
15 derived samples wherein the presence of cFAP is unknown and/or where such presence is to be excluded.

As used herein, subject-derived preparations also include tissue preparations. In particular, the invention provides methods and compositions for the immunohistochemical analysis of
20 such tissue preparations, for tissue slides prepared according to standard methods known in the art. It is preferred that the sample for immunohistochemical analysis according to the methods of the invention are OCT snap frozen.

25 5.4.2 Pharmaceutical compositions

The anti-cFAP antigen binding molecules of the invention, and their methods of production and use, are provided not only as diagnostic tools but are also envisioned to have applicability in the treatment and amelioration of disease and disease symptoms, as well as applicability in model systems for investigating disease therapies, specifically diseases characterized by cFAP
30 expression. Accordingly, the invention provides pharmaceutical compositions comprising one or more pharmaceutically acceptable carriers and (i) a specific and selective anti-cFAP antigen binding molecule as disclosed herein; (ii) a polynucleotide encoding an antigen binding

molecule of (i); (iii) a vector comprising a polynucleotide of (ii); or (iv) a host cell comprising a polynucleotide of (ii) and/or a vector of (iii) that expresses antigen binding molecule of (i).

5 The pharmaceutical composition disclosed herein are formulated to be suitable for administration to a human or animal subject. In the manufacture of a pharmaceutical formulation, the antibodies or antigen binding fragments of the invention are admixed with a pharmaceutically acceptable carrier, excipient, and/or diluents. The carrier, excipient and/or diluent must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the subject. Examples of suitable
10 pharmaceutical carriers for use with antibody-based compositions are well known in the art and can be formulated by conventional methods.

The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms
15 of completely or partially preventing a disease or symptom thereof, and/or may be therapeutic in terms of partially or completely curing the disease or condition, and/or adverse effect attributed to the disease or condition. The term "treatment" as used herein covers any treatment of a disease or condition in a subject and includes: (a) preventing and/or ameliorating a disease or condition (or symptom thereof) from occurring in a subject, where
20 the subject is known or believed to have the disease or condition, or is predisposed develop the disease or condition; (b) inhibiting the disease or condition, i.e., arresting its development, such as inhibition of disease progression; (c) relieving the disease or condition, i.e. causing regression of the disease; and/or (d) preventing, inhibiting or relieving any symptom or adverse effect associated with the disease or condition. Preferably, the term "treatment" as
25 used herein relates to medical intervention of an already manifested disorder, e.g., the treatment of a diagnosed disease or condition characterized by cFAP expression. The term "treatment" also includes the amelioration, e.g. improvement or lessening of, of one or more indicators, signs, or symptoms of a disease, disorder, or condition characterized by cFAP expression. Thus, the term "treatment" can include a delay or slowing in the progression or
30 severity of one or more indicators of a condition or disease characterized by cFAP expression. The progression or severity of indicators may be determined by subjective or objective measures, which are known to those skilled in the art.

The term “prevent” as used herein refers to delaying or forestalling the onset, development or progression of a disease, disorder, or condition characterized by cFAP expression for a period of time from minutes to indefinitely.

5

The subject to be treated by the methods and uses disclosed herein can be a mammal, including, but not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, horses and birds), rabbits, rodents (e.g., mice and rats) and primates (e.g., humans and non-human primates such as monkeys). It is preferred that the subject to be treated is a human. In particular, the subject to be treated, e.g. a human patient, is at risk for, is suspected to have, or has been determined to have a disease or condition characterized by cFAP expression as described herein or as known in the art.

10

5.5 Kits

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The invention also provides a kit comprising any manufacture (e.g. a package or container) comprising at least one reagent of the present invention, i.e. one or more of (i) an antigen binding molecule of the invention, (ii) a nucleic acid molecule of the invention, (iii) a vector of the invention, (iv) a host cell of the invention, and/or (v) an antigen binding molecule produced or obtained by a method of the invention. The kit may be promoted, distributed, or sold as a unit for performing the methods of the present invention.

20

The specific and selective anti-cFAP antigen binding molecule as disclosed herein can be used in cFAP detection kit. Such a detection kit may contain a capturing reagent, a detecting reagent and/or a solid phase. The anti-cFAP antigen binding molecule of the invention may or may not be conjugated or otherwise linked to the solid phase (e.g. magnetic microbeads or plate). Further the anti-cFAP antigen binding molecule of the invention may or may not be detectably labeled. The anti-cFAP antigen binding molecule of the invention can be used as the capturing reagent or the detecting reagent. The detecting reagent is preferably labeled. In such a case, the kit may additionally contain substrates and /or reagents allowing the detection of the label.

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The detection kit may additionally contain a pretreated solution of a biological sample as a positive or negative control (e.g. a solution comprising cFAP), a washing solution, and/or calibration standards.

5 In the foregoing detailed description of the invention, a number of individual elements, characterizing features, techniques and/or steps are disclosed. It is readily recognized that each of these has benefit not only individually when considered or used alone, but also when considered and used in combination with one another. Accordingly, to avoid exceedingly repetitious and redundant passages, this description has refrained from reiterating every
10 possible combination and permutation. Nevertheless, whether expressly recited or not, it is understood that such combinations are entirely within the scope of the presently disclosed subject matter.

All technical and scientific terms used herein, unless otherwise defined, are intended to have
15 the same meaning as commonly understood by one of ordinary skill in the art. Reference to techniques employed herein are intended to refer to the techniques as commonly understood in the art, including variations on those techniques or substitutions of equivalent techniques that would be apparent to one of skill in the art.

20 All amino acid sequences provided herein are presented starting with the most N-terminal residue and ending with the most C-terminal residue (N→C), as customarily done in the art, and the one-letter or three-letter code abbreviations as used to identify amino acids throughout the present invention correspond to those commonly used for amino acids.

25 In this specification, a number of documents including patent applications and manufacturer's manuals are cited. The disclosure of these documents, while not considered relevant for the patentability of this invention, is herewith incorporated by reference in its entirety. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by
30 reference.

In addition, the present invention relates to the following:

1. An antigen-binding molecule specific for membrane bound fibroblast activation protein (FAP) as expressed on the surface of a cell (cFAP), which antigen binding molecule discriminates between said cFAP and an extracellular soluble non-membrane associated fibroblast activation protein (sFAP).
5
 2. The antigen-binding molecule according to item 1 wherein said cFAP has the amino acid sequence of SEQ ID NO:1 and is expressed on the surface of a HEK293 cell, and wherein said sFAP has an amino acid sequence of SEQ ID NO:2 or is as present in human plasma (spFAP).
10
 3. The antigen-binding molecule according to item 1 or 2, wherein said molecule has an EC₅₀ for binding to cFAP of less than 5nM and an EC₅₀ for binding to sFAP that is at least 20 fold weaker.
15
 4. The antigen-binding molecule according to item 3, wherein said EC₅₀ for binding to sFAP is determined as the EC₅₀ for binding to human plasma known or believed to contain spFAP.
20
 5. The antigen-binding molecule according to item 3 or 4 , wherein
 - (i) the EC₅₀ of said molecule for binding to spFAP is not detectable or incalculable when assessed in an assay, in which assay, and where assessed under the same conditions, the EC₅₀ of sibrotuzumab or 4B9 for binding to said spFAP is <1nM or <10nM, respectively;
25
- and
- (ii) said antigen-binding molecule has one or more of the following cFAP binding properties determined by flow cytometry:
 - (a) an apparent K_d of binding to said cell expressing said cFAP of less than 2nM;
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 - (b) an EC₅₀ of binding to said cell expressing said cFAP of less than 2 nM;
 - (c) an EC₅₀ of binding to said cell expressing said cFAP that is within at least 1-fold of the EC₅₀ of sibrotuzumab or 4B9 assessed under the same conditions;
35
 - (d) a maximum binding to said cell expressing said cFAP that is within 1-fold of sibrotuzumab or 4B9 assessed under the same conditions.

6. The antigen-binding molecule according to any one of items 3 to 5, wherein the ratio of binding to cFAP in the presence of undiluted human plasma having an spFAP concentration of at least 80ng/mL relative to the binding in the absence of said undiluted human plasma is at least 80% at an antigen-binding molecule concentration of 0.8nM, at least 60% at an antigen-binding molecule concentration of 0.16 nM, or at least 50% at an antigen-binding molecule concentration of 0.0032 nM; wherein as assessed in the same assay and under the same conditions,
- 5 (i) the ratio of binding for 4B9 to cFAP in the presence of said undiluted human plasma relative to the binding in the absence of said undiluted human plasma is less than 10% at a 4B9 concentration below 0.8nM; or
- 10 (ii) the ratio of binding for sibrotuzumab to cFAP in the presence of said undiluted human plasma relative to the binding in the absence of said undiluted human plasma is less than 25% below 0.8nM.
- 15 7. The antigen-binding molecule according to any one of items 1 to 6, which molecule comprises or consists of an antibody, an antibody antigen-binding domain or an antibody antigen-binding fragment.
8. The antigen-binding molecule according to item 7, wherein said molecule comprises more than one antibody, antibody antigen-binding domain, or antibody antigen-binding fragment.
- 20 9. The antigen-binding molecule according to item 8 comprising more than one antibody antigen-binding domains or more than one antibody antigen-binding fragments, which more than one antibody antigen-binding domains or antibody antigen-binding fragments have the same amino acid sequence.
- 25 10. The antigen-binding molecule according to item 8 comprising more than one antibody antigen-binding domains or more than one antibody antigen-binding fragments, which more than one antibody antigen-binding domains or fragments specifically bind the same antigen and have different amino acid sequences.
- 30 11. The antigen-binding molecule according to any one of items 7 to 10 comprising at least one antibody antigen-binding domain or fragment that is an Fab, Fab', F(ab')₂, Fv, scFv, or a single domain binding fragment.
- 35 12. The antigen-binding molecule according to item 11, wherein said single domain binding fragment is a sdAbs, dAb, nanobody or V_HH domain.

13. The antigen-binding molecule according to any one of items 1 to 11, wherein said molecule comprises a human Fc domain.
14. The antigen-binding molecule according to item 13, wherein said Fc domain (i) comprises the mutation S239D/A303L/I332E according to EU numbering (the DEL modification), (ii) is afucosylated, or (iii) is modified to increase ADCC or ADCP function relative to the same antigen-binding molecule comprising an IgG1 Fc domain having the amino acid sequence of SEQ ID NO:22.
15. The antigen-binding molecule according to any one of items 7 to 14, wherein said antibody, antibody antigen-binding domain or antibody antigen-binding fragment is a human, chimeric, humanised, CDR-grafted, and/or deimmunised antibody, antibody antigen-binding domain or antibody antigen-binding fragment.
16. The antigen-binding molecule according to any one of items 1 to 15, wherein said molecule comprises or consists of one or more V_HH domains.
17. The antigen-binding molecule according to item 16, wherein said one or more V_HH domains comprise
 - (i) a CDR1 having the sequence of X₁X₂TX₃X₄X₅YAX₆G (SEQ ID NO:3), wherein X₁ is A, G or S; wherein X₂ is P, R or G; wherein X₃ is K, F, R or N; wherein X₄ is S, R or G; wherein X₅ is T, S or N; and wherein X₆ is M, I or L;
 - (ii) a CDR2 having the sequence of X₁IX₂WX₃X₄X₅X₆TX₇YX₈DSVKG (SEQ ID NO:4), wherein X₁ is A or V; wherein X₂ is N, W or S; wherein X₃ is S or A; wherein X₄ is G or N; wherein X₅ is T or G; wherein X₆ is I, L, S, T or V; wherein X₇ is S, Q or N; and wherein X₈ is S or T; and/or
 - (iii) a CDR3 having the sequence of AADX₁DFRTVGSRRPSY (SEQ ID NO:5), wherein X₁ is R, S or K;
 and wherein said CDRs are defined according to IMGT numbering.
18. The antigen-binding molecule according to item 16 or 17, wherein said one or more V_HH domain comprises
 - (i) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7);
 - (ii) a CDR2 having the sequence of VINWSGTLTSYADSVKG (SEQ ID NO:8), VINWAGTLTSYADSVKG (SEQ ID NO:9), VISWSGTLTSYADSVKG (SEQ ID NO:10),

AISWSGGTTQYTSVK (SEQ ID NO:11), AISWSGGTTNYTSVK (SEQ ID NO:12),
or AISWSGGTTNYADSVK (SEQ ID NO:13); and/or

(iii) a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14).

and wherein said CDRs are defined according to IMGT numbering.

5

19. The antigen-binding molecule according to any one of items 16 to 18, wherein said one or more V_HH domains comprise
- (i) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VINWSGTLTSYADSVK (SEQ ID NO:8) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (ii) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VINWAGTLTSYADSVK (SEQ ID NO:9) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (iii) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VISWSGTLTSYADSVK (SEQ ID NO:10) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (iv) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTQYTSVK (SEQ ID NO:11) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14);
- (v) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTNYTSVK (SEQ ID NO:12) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14) ; or
- (vi) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTNYADSVK (SEQ ID NO:13) and a CDR3 having the sequence of AADRDFRTVGSRPSY (SEQ ID NO:14) .

25

20. The antigen-binding molecule according to any one of items 16 to 19, wherein said one or more V_HH domains comprise the sequence of
- (i) QVQLVESGGGLVQAGDSLSCVASAPTRSTYAMGWFRQAPGKEREFAAVINWSGTLTSYADSVKGRFTISRDNKNTVFLQMNSLKPDDTAVYYCAADRDFRTVGSRPSYWGQGTPVTVSS (SEQ ID NO:15);

30

- (ii) QVQLVESGGGLVEPGDSLRLSCAASGRTFSSYAIGWFRQAPGKEREFVAAISWSGGTT
NYTDSVKGRFTISRDNKNTVYLQMNSLKPDDTAVYYCAADRDFRTVGSRPSYWSKGT
RVTVSS (SEQ ID NO:16);
- (iii) QVQLVESGGGLVQPGGSLRLSCSASAPTRSTYAMGWFRQAPGKEREFASVISWSGTLT
SYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGQGT
TVTVSS (SEQ ID NO:17);
- (iv) QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISWSGGTT
QYTDSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGQGT
TVTVSS (SEQ ID NO:18);
- (v) QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISWSGGTTN
YADSVKGRFTISRDNKNTLYLQMNSLRPEDTAVYYCAADRDFRTVGSRPSYWGQGT
TVTVSS (SEQ ID NO:19).
21. The antigen-binding molecule according to any one of items 16 to 19, wherein said one
or more V_H domain comprises a variant amino acid sequence having at least 85%,
90% or 95% sequence identity to any one of SEQ ID NOs:15 to 19, wherein said variant
is the modification of any one of SEQ ID NOs:15 to 19 by one or more conservative
amino acid substitutions.
22. The antigen-binding molecule according to item 21, wherein said conservative amino
acid substitution is the substitution of an amino acid with another amino acid selected
from its same group, wherein the groups of amino acids are
- the nonpolar, hydrophobic amino acids consisting of Gly, Ala, Val, Leu, Ile, Phe,
Tyr, Trp, and Met;
 - the polar, neutral amino acids consisting of Ser, Thr, Asn, and Gln;
 - the positively charged, basic amino acids consisting of Arg, Lys, and His, and
 - the negatively charged, acidic amino acids consisting of Asp and Glu
- wherein if Cys is to be conservatively substituted, it is substituted with Ser or Ala, and
wherein if Pro is to be conservatively substituted it is substituted with Ala.
23. A polynucleotide encoding the antigen-binding molecule according to any one of items
1 to 22.
24. A vector comprising the polynucleotide according to item 23.

25. A host cell comprising the polynucleotide according to item 23, or the vector according to item 24.
- 5 26. A method of producing the antigen-binding molecule according to any one of items 1 to 22 comprising culturing the host cell according to item 25 and isolating said antigen-binding molecule.
- 10 27. A composition comprising (i) the antigen-binding molecule according to any one of items 1 to 22 or obtainable by the method of item 26, (ii) the polynucleotide according to item 23, (iii) the vector according to item 24, or (iv) the host cell according to item 25.
- 15 28. The antigen-binding molecule according to any one of items 1 to 22 or obtainable by the method of item 26, or the composition according to item 27 for use as a medicament.
- 20 29. A pharmaceutical composition comprising the antigen-binding molecule according to any one of items 1 to 22 or obtainable by the method of item 26, or the composition according to item 27.
- 25 30. The antigen-binding molecule according to any one of items 1 to 22 or obtainable by the method of item 26, or the composition according to item 27 for use in the treatment of a disease characterized by the overexpression of cFAP.
- 30 31. A method for the treatment of a disease characterized by the overexpression of cFAP comprising administering to a subject in need thereof a therapeutically effective amount of the antigen-binding molecule according to any one of items 1 to 22 or obtainable by the method of item 26, or the composition according to item 27.
32. The antigen-binding molecule according for use according to item 30, wherein said treatment is for use in a subject having a blood spFAP concentration of greater than 40 ng/mL.

33. The method according to item 31, wherein said subject has a blood spFAP concentration of greater than 40 ng/mL.
34. The antigen-binding molecule according to any one of items 1 to 22 or obtainable by the method of item 26, or the composition according to item 27 which is a diagnostic for use *in vitro* or *in vivo*
- 5 (i) for the detection of cFAP; and/or
- (ii) for discriminating cFAP from spFAP in serum, plasma, tissue or bodily fluids.
- 10 35. A kit comprising the antigen-binding molecule according to any one of items 1 to 22 or obtainable by the method of item 26.

6. EXAMPLES

15 6.1 Materials and methods

The description of the following materials and methods is generally applicable to the examples herein, except as otherwise noted.

20 6.1.1 Primary cells

Human uterine/endometrial fibroblasts (HUF), human pulmonary fibroblast (HPF), human cardiac fibroblasts (HCF) were used as primary cells (Sigma Aldrich/Merck/Promocell). Additionally, the cell lines human dermal fibroblasts (Detroit 551) and HEK293 were used. The

25 HUF and HPF were cultured in fibroblast growth media 2 (with supplements) (PromoCell GmbH; Heidelberg, Germany). HCF were cultured in Fibroblast growth media 3 (with supplements) (PromoCell). Detroit 551 was cultured in Eagle's Minimum Essential Medium (Catalog No. 30-2003) completed with fetal bovine serum to a final concentration of 10%.

30 6.1.2 Human plasma samples

Human plasma was obtained from fresh female EDTA-treated blood and stored at 4°C up to 24 hours until it was processed. The blood was centrifuged at 2000 rpm for 10 minutes to separate the plasma layer. The plasma was then filtered first through a 40 µm sieve and subsequently

through a 0.45 µm filter. If immediate usage was not required, the plasma was aliquoted into 5 ml volumes and stored at -80°C. All plasma handling was done on ice under BSL2 conditions.

6.1.3 Human FAP ELISA

5

The following FAP ELISAs were used in the working examples detailed below.

- a) Human FAP DuoSet ELISA (Biotechne, DY3715) was used to quantify human spFAP in plasma, protocol according to the manufacturer's recommendations.
- b) Forward Plasma ELISA was used to quantify the affinity of the antigen binding molecules to spFAP. Human FAP DuoSet ELISA kit (Biotechne, DY3715) was used where the capture antibody was replaced with the antigen binding molecules in serially diluted concentrations. The EC50 of the antigen binding molecules to human spFAP was assessed.
- c) Reverse Plasma ELISA was used to quantify and verify the affinity of the antigen binding molecules to spFAP with an alternative orthogonal setup. Human FAP DuoSet ELISA kit (Biotechne, DY3715) was used, where the detection antibody was replaced with the antigen binding molecules of the invention in serially diluted concentrations. The EC50 of the antigen binding molecules to human spFAP was assessed. The antigen binding molecules of the invention were detected with a biotinylated anti-human IgG1 Fc antibody (use 1:1000, B6775 Merck/Sigma Aldrich) and Streptavidin-HRP (e.g., DY998 Bio-Techne), or suitable replacement.
- c) Recombinant FAP ELISA. Protocol was established in house for EC50 measurement of the antigen binding molecules to recombinant FAP (rFAP; SEQ NO:2) where the plate was coated with rFAP at 10µg/ml or 5 µg/ml overnight at 4°C.

FAP in plasma and/or in cell culture was measured using the Human FAP DuoSet ELISA kit (Biotechne, DY3715) and high-binding 96-well plates (Corning, Ref. 3590) according to manufacturer's guidelines as detailed in (a).

To measure the binding of the antigen-binding molecules of the invention (also referenced as “antibody constructs” herein) to plasma (including the antibody construct ab1-hFc and ab3-hFc as detailed below), forward and reverse ELISAs for FAP were established as described in (b) and (c). Forward and reverse ELISA were used to exclude lower binding due to competition
5 with other antibodies used in the same assay (e.g. the coating antibody, which overlaps in binding with the ab1-hFc and ab3-hFc used as detection antibody).

The forward plasma FAP ELISA were performed with plates coated with antigen-binding molecules of the invention serving as capture antibodies, and a detection antibody obtained
10 from the DY3715 Human FAP ELISA kit. The assay was performed in high-binding 96-well EIA/RIA plates (Corning) using a coating buffer of 1x PBS, a wash buffer, a freshly prepared and sterile-filtered dilution buffer consisting of PBS with 1% BSA, human plasma (see above), Streptavidin-HRP (DY998 Bio-Techne, dilution according to the manufacturer), HRP substrate (for example, 1 Step Ultra TMB ELISA Thermo scientific Ref:34028), ELISA stop solution
15 (Invitrogen SS04), and a plate sealer.

For the coating of the ELISA plates, serial dilutions of antigen-binding molecules of the invention were prepared in PBS. A 96-well high-binding plate from Corning was then coated with 100 µL per well of the dilutions, immediately sealed with a plate sealer, and incubated
20 overnight at 4°C. Next, each well was washed three times with 300 µl of wash buffer (e.g. Thermo scientific Cat. No: J63596.K2 diluted in dH₂O directly before use) with complete removal of liquid after each step. The plate was then blocked by adding 200 µl of Reagent Diluent (such as PBS with 1 % BSA, protease free (Biowest P6155-100g or Sigma Aldrich A3059-50g)) to each well, followed by an incubation period of 1 to 2 hours at room temperature. After
25 another round of washing, 100 µl of the prepared human plasma was added to each well and the plate covered and incubated for 2 hours at room temperature. Then, 100 µl of Detection Antibody diluted to 200 ng/ml was added to each well and incubated for 2 hours. Subsequently, 100 µl of a working dilution of Streptavidin-HRP was added to each well, covered, and incubated for 20 minutes avoiding exposure to direct light. After another washing
30 step, 100 µl of HRP substrate was added to each well. Following an incubation period of up to 20 minutes, the resulting signal was monitored. Finally, 100 µl of Stop Solution was added to each well and mixed gently by tapping. The optical density of each well was measured

immediately at 450 nm with a microplate reader. If wavelength correction was available, readings at 540 nm or 570 nm were subtracted from the initial 450 nm readings.

In the reverse plasma FAP ELISA, the standard procedure was modified by substituting the detection antibody from the Human FAP DuoSet ELISA Kit with antigen-binding molecules of the invention at various serially diluted concentrations. Additionally, the biotinylated anti-human IgG1 (B6775 from Sigma Aldrich) was used for detection in combination with Streptavidin-HRP (DY998 Bio-Techne). The manufacturer's recommended protocol for the FAP DuoSet ELISA kit was used for the remaining steps of the procedure.

6.1.4 Cell binding assay by flow cytometry

HEK 293 cells were transfected with DNA encoding whole human Fibroblast Activation Protein (FAP, SEQ ID NO: 1) with lipofectamine 2000 (Invitrogen) according to the manufacturer standard protocol.

100,000 transfected cells were plated in each well. Primary antibodies were serially diluted in a solution of PBS and 1% Bovine Serum Albumin (BSA). The primary antibodies were then added to the respective wells and incubated at a temperature of 4°C for one hour. The incubation period allowed for sufficient binding of the primary antibodies to the target antigens on the cells.

Subsequently, after washing, a secondary antibody staining was performed. Briefly, a 1:100 dilution of an anti-human Fc antibody conjugated with R-Phycoerythrin (RPE) in 1% BSA was added to the wells containing the cells and primary antibodies, followed by incubation for 30 minutes at 4°C and two washing steps in PBS and 1% BSA. Flow cytometry was then performed using standard procedures.

6.1.5 Cross-Species Reactivity to murine and cynomolgus FAP

The cross-species reactivity of FAP antigen binding molecules was measured against murine and cynomolgus (cyno) FAP proteins having the amino acid sequences (SEQ ID NO: 23 or 24,

respectively) as transiently expressed on HEK293 cells according to standard protocols. The subsequent steps of the protocol were those outlined in section 6.1.4.

6.1.6 Competitive cell binders flow cytometry in the presence of soluble FAP (plasma)

5

Antigen-binding constructs according to the invention comprising murine IgG2a Fc regions were incubated in human plasma for one hour at room temperature or at 37°C. Next, the human plasma containing the antigen-binding constructs was added to transfected cells expressing complete human FAP protein (SEQ ID NO:1) at a concentration ranging from 50,000
10 to 100,000 cells per well and incubated for 30 minutes at 4°C. Next, the cells are washed and subsequently stained with fluorescently labelled anti-mouse IgG. The binding was quantified by measuring the Mean Fluorescence Intensity (MFI) by flow cytometry.

6.1.7 FAP enzyme activity assay

15

FAP enzyme activity was assayed in 20 mM Tris/HCl, 0.1 M NaCl, 1 mM EDTA in the presence of 50µM Suc-Gly-Pro-Leu-Gly-Pro-AMC (#I-1350.0100, BACHEM, Bubendorf, Switzerland) in black 96 well plates. Donor plasma and soluble FAP with enzymatic activity (Acro Biosystems) at 0.4 ug/ml were used in a total volume of 100 µl (separately). The reaction mixture was
20 incubated for 60 min at 37°C, and the fluorescence of AMC released by FAP-induced cleavage was determined at an emission wavelength of 475 nm (excitation 380 nm) with a plate reader.

6.1.8 ADCC (Antibody-dependent cell-mediated cytotoxicity) assay

25 The ADCC Reporter Bioassay, Core Kit (Promega Cat. # G7010 and G7018) was used for quantifying biological activity on pathway activation by antigen binding molecules according to the present invention.

On Day 1, target cells were plated in a white 96 well plate (TC treated) (20k cells/well) in cell
30 culture media. On Day 2, antigen binding molecules were diluted in assay media (without FBS). The culture media was removed from target cells on the 96 well plate and 50 µl of serially diluted antigen binding molecules were added to each well. The antigen binding molecules

were then incubated for 20 min, followed by the addition of 120k cells/well effector cells in 1% low IgG FBS in assay media. Next, the cells were incubated for 18h at 37°C. On Day 3, the plate was equilibrated at RT for 10 min, Bio-Glow was then added to each well (75µl) and incubated for 5 min for luminescence measurement according to manufacturer guidelines.

5

ADCC in presence of rFAP:

The assay is performed as described above, with the exception that the antigen binding molecules are serially diluted in assay media (without FBS) containing spFAP.

10 6.1.9 ADCP (Antibody-dependent cell-mediated phagocytosis) assay

The ADCP Reporter Bioassay reflects the mechanism of action of biologics designed to bind and activate FcγRIIIa. The bioluminescent cell-based assay (Promega Cat. # G9871) was used according to manufacturer guidelines to measure the potency and stability of the antigen binding molecules fused to human FC that specifically bind and activate FcγRIIIa.

15

The assay utilized a genetically engineered Jurkat T cell line that expresses human FcγRIIIa and a luciferase reporter driven by an NFAT-response element (NFAT-RE). Briefly, on day 1, target cells were plated in white 96 well plate (20k cells/well) in cell culture media. On day 2, antigen binding molecules were diluted in assay media (without FBS). Next, the media was removed from cells, 50 µl of serially diluted antigen binding molecules were added to each well and incubated for 20 min. Afterwards, 120k effector cells/well in assay media supplemented with 8% low IgG FBS in 50 µl (total volume 100 µl) were added and incubated for 18h-20h at 37°C. On day 3, the 96 well plate was equilibrated at room temperature for 10 min, Bio-Glow was then added to each well (75 µl) and incubated for 5 min. for luminescence measurement according to manufacturer guidelines.

20

25

6.1.10 Antigen binding molecule internalization assay on FAP expressing cells

WI-38 cells were used to assess the internalization of antigen binding molecules according to the invention. Antibodies were 4-fold serial diluted in assay buffer starting from 200 nM. Ten thousand WI-38 cells per well (96 well plate) were seeded and grown overnight at 37°C. The

30

antigen binding molecules were added to the cells and incubated for 1 hour at 4°C. This was followed by a wash and a subsequent 30-minute incubation with pHrodo-labelled secondary antibodies at 4°C. After another washing step, the internalization process was activated by incubating the cells at 37°C for 4 hours and 24 hours. At the end of the incubation period, the cells were stained with Hoechst and Calcein AM for 15 minutes at 37°C. The Operetta system was used for the readout, specifically calculating the number of pHrodo spots per cell.

6.1.11 *Binding to human DPP4*

The reactivity of FAP antigen binding molecules was measured against human DPP4, which is a close homologue of FAP. HEK 293F cells were transfected with DNA encoding whole human DPP4 (SEQ ID NO: 34) with lipofectamine 2000 (Invitrogen) according to standard protocols. The subsequent steps of the protocol were those outlined in section 6.1.4.

6.1.12 *In-vivo imaging*

Female C57BL/6 mice, aged 8 weeks, were inoculated subcutaneously (SC) with MC38-hFAP cells at a 1×10^6 cells per animal. 28 days post inoculation, and 24 hours prior to sacrifice, mice were administered either 7.5 mg/kg of Ab3z7p2-Scarlett, a fluorescently labelled Scarlett-conjugate of Ab3, or PBS as a control, via the intraperitoneal (IP) route. Fluorescence was measured at different timepoints post inoculation (3h, 24h). Fluorescent imaging was performed using an imaging system equipped to detect the Scarlett fluorophore. Imaging parameters included an excitation wavelength range of 570–604 nm and an emission wavelength range of 620–639 nm. Fluorescence data were analyzed visually to demonstrate specific tumor uptake.

6.1.13 *ADCP assay with human macrophages*

The ADCP assay utilizes monocyte-derived macrophages, isolated from primary cells from healthy human blood donors. CD14⁺ monocytes were enriched from peripheral blood mononuclear cells (PBMCs) and subsequently differentiated into macrophages by culturing in the presence of recombinant human macrophage colony-stimulating factor (rhM-CSF). The assay measures the phagocytic activity of macrophages, specifically evaluating the

internalization of antibody-opsonized target cells. This quantification is achieved through fluorescence-based flow cytometry, which tracks the uptake of fluorescently labeled target cells by macrophages.

5 *Effector cell preparation*

CD14⁺ monocytes were isolated from PBMCs and differentiated into macrophages by culturing in RPMI-1640 medium supplemented with 50 ng/mL recombinant human macrophage colony-stimulating factor (rhM-CSF) for 6–8 days at 37°C with 5% CO₂.

10 *Target cell preparation*

HT1080-hFAP target cells were labeled with 1 nM CFSE (carboxyfluorescein succinimidyl ester) in the dark at 37°C for 20 minutes and washed in PBS.

Antibody Preparation

- 15 Antibodies were prepared at varying concentrations, starting from 5 nM and diluted serially 8-fold in PBS.

Co-Culture and Phagocytosis Assay

- 20 Target cells were pre-incubated with the prepared antibody dilutions for 15–20 minutes at room temperature. Subsequently, the target cells were co-cultured with macrophages at an effector-to-target (E:T) ratio of 1:1. The co-culture was maintained for 4 hours at 37°C in a humidified atmosphere with 5% CO₂.

Secondary Antibody Staining

- 25 After the co-culture, cells were stained with CD14-APC antibody (1:150 dilution) in 1% bovine serum albumin (BSA) at a volume of 100 µL per well. The staining was performed on ice for 40 minutes.

Flow Cytometry Analysis

- 30 The samples were washed and resuspended in PBS before being analyzed via fluorescence-activated cell sorting (FACS). FACS analysis was used to measure phagocytosis by detecting CFSE-labeled target cells internalized by CD14⁺ macrophages.

6.1.14 FDA tissue staining

Materials

- 5 Tissue Array: FDA standard frozen tissue array (Catalog No. T6234701-2, AMSBIO).
Primary Antibody: ab3-mFc (murine IgG2a).
Stock concentration: 1.72 mg/ml.
Dilution: 1:8000.
Negative control: Mouse IgG2A Isotype Control, Biotechne (Catalog No. MAB0031).
10 Blocking: BloxAll Blocking Reagent and Peroxide block.

The human FDA tissue array (AMS-Bio) included fresh frozen tissue slides for the major human organs from 3 distinct donors of mixed ages and genders. All slides were stained by IHC with ab3-mFc, scanned and quantified by AI algorithms for digital pathology (Qpath). Briefly, the
15 fresh frozen tissue slides were air-dried overnight at room temperature and subsequently fixed in acetone at 4°C for 10 minutes. After fixation, the sections were stained using the Leica Bond system following the HRP Refine 60 Kryo + BloxAll protocol. For detection, the BOND Refine Detection System (DS9800) was used. The antibody dilution was ab3-mFc 1:8000 of 1.72 mg/ml, the control antibody was diluted accordingly.

20

6.2 Example 1 - Generation of cFAP specific and selective antigen-binding molecules

An alpaca was subcutaneously immunized 4 times with a mixture of different FAP species, including membrane proximal truncated peptides of different lengths: full-length human FAP
25 (SEQ ID NO:1), a human FAP fragment spanning amino acids 20-45 of human FAP (SEQ ID NO:25), a human FAP fragment spanning amino acids 24-50 of human FAP (SEQ ID NO:26) fused to KLH, recombinant cynomolgus FAP (Accession # XP_005573377.1, SEQ ID NO: 27) and recombinant murine FAP (Accession # P97321; SEQ ID NO: 28).

30 PBMCs from immunized Alpaca were isolated and a phage display library generated according to standard protocols. Panning was performed using human primary cells overexpressing cellular FAP (cFAP) including HUF, HPF, HCF and the dermal cell line Detroit551. Negative

selection steps were implemented against human plasma to eliminate binders against soluble FAP (spFAP). Multiple rounds of positive and negative selection using the previously defined human primary cells and human plasma were performed to enrich for highly specific cFAP binders lacking binding to spFAP. Specific cFAP binders were verified for the lack of spFAP testing for binding to human plasma by ELISA (forward FAP plasma ELISA).

The selected V_HH antibodies that specifically and selectively bound cFAP over spFAP were subsequently humanized according to Sulea, Methods Mol. Biol. 2446(2022), 299-312. Additional mutations were introduced to improve safety and scalability for commercial production by removing sites with potential post translational modification liabilities. Obtained and modified V_HH antibodies were sequenced and aligned according to IMGT numbering, and characterized by the following IMGT CDR sequences:

- (i) a CDR1 having the sequence of X₁X₂TX₃X₄X₅YAX₆G (SEQ ID NO: 3), wherein X₁ is A, G or S; wherein X₂ is P, R or G; wherein X₃ is K, F, R or N; wherein X₄ is S, R or G; wherein X₅ is T, S or N; and wherein X₆ is M, I or L;
- (ii) a CDR2 having the sequence of X₁IX₂WX₃X₄X₅X₆TX₇YX₈DSVKG (SEQ ID NO:4), wherein X₁ is A or V; wherein X₂ is N, W or S; wherein X₃ is S or A; wherein X₄ is G or N; wherein X₅ is T or G; wherein X₆ is I, L, S, T or V; wherein X₇ is S, Q or N; and wherein X₈ is S or T; and
- (iii) a CDR 3 having the sequence of AADX₁DFRTVGSRPYS (SEQ ID NO:5), wherein is R, S or K.

Exemplary antigen-binding molecules according to the above include the specific and selective anti-cFAP camelid V_HH antibodies ab1 and ab3, and the humanized and optimized variants sequences ab1z12, ab3z7 and ab3z10 as set forth in Table 1, below.

Table 1:

Name	Sequence	SEQ ID NO:
ab1	QVQLVESGGGLVQAGDSLSCVASAPTRSTYAMGWFRQAPGKEREFAAVINWSGTLTSYADSVKGRFTISRDNKNTVFLQMNSLKPDDTAVYYCAADRDFRTVGSRPSYWGGQTPVTVSS	15
ab3	QVQLVESGGGLVEPGDSLRLSCAASGRTFSSYAIGWFRQAPGKEREFAAISWSGGTTNYTDSVKGRFTISRDNKNTVYLQMNSLKPDDTAVYYCAADRDFRTVGSRPSYWSKGRTRTVSS	16
ab1z12	QVQLVESGGGLVQPGGSLRLSCSASAPTRSTYAMGWFRQAPGKEREFAVISWSGTLTSYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGGQTTVTVSS	17
ab3z7	QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFAAISWSGGTTQYTDVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGGQTTVTVSS	18
ab3z10	QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFAAISWSGGTTNYADSVKGRFTISRDNKNTLYLQMNSLRPEDTAVYYCAADRDFRTVGSRPSYWGGQTTVTVSS	19

6.3 Example 2 – Selective binding to cFAP over spFAP

- 5 The humanized and optimized V_HH sequences of Example 1 were formatted into antigen-binding Fc-region containing constructs by linking the sequences to an IgG1 Fc domain using a GS linker according to Table 2 (the “antigen-binding” constructs are also termed “antibody” constructs herein).

10 Table 2

Name	Sequence	SEQ ID NO:
ab1z12-hFc	QVQLVESGGGLVQPGGSLRLSCSASAPTRSTYAMGWFRQAPGKEREFAVISWSGTLTSYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGGQTTVTVSSGSEPKSCDKTHTCPPCPAPELGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK	29

ab3z7-hFc	QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFV SAISWSGGTTQYTDVSKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCA ADRD FRTVGS RPSYWGQGTTVT VSSGSEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPGK	30
ab3z10-hFc	QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFV SAISWSGGTTNYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCA ADRD FRTVGS RPSYWGQGTTVT VSSGSEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPGK	31

The constructs were assessed for the selective binding of cFAP over spFAP (i.e. FAP as present in human plasma). A sandwich forward and reverse ELISA (as described in the Materials and Methods section, above) was used with samples of human plasma or HEK293 cells transiently transfected to express cFAP. The commercially available anti-FAP antibodies 4B9 (WO 2020/198665A1) comprising the same IgG1 Fc domain listed above and sibrotuzumab (WO2002083171A2) were also tested for comparison; isotype control was used as negative control.

The results for binding to spFAP are shown in Figure 1 and reported in Table 3.

Table 3: Binding affinities for soluble FAP as present in human plasma (spFAP)

Antibody	EC_{50} (nM)	$Max\ OD_{450} - OD_{540}$
ab1z12-hFc	NA	2.9
ab3z7-hFc	NA	0.25
ab3z10-hFc	NA	0.31
4B9-hFc	8.8	3.3
Sibrotuzumab	0.47	3.3

* NA – not detectable/incalculable

The EC_{50} values of the tested humanized antibody constructs, including variants of ab1 and ab3, had incalculable EC_{50} up to 100 nM for FAP in human plasma (spFAP). Thus, these constructs did not bind or bound with only very low affinity to spFAP. In contrast, control

antibodies 4B9 and sibrotuzumab exhibited high binding affinities to spFAP with EC50 values of approximately 0.5 nM and 8.8 nM, respectively (Figure 1).

5 The constructs were also tested for binding to cFAP using HEK 293F cells transiently transfected with the complete FAP sequence (SEQ ID NO: 1) in a cell binding assay and using flowcytometry. The EC50 of the tested humanized antibody constructs to cFAP is comparable to that of control antibodies 4B9 and sibrotuzumab (all tested antibodies/antibody constructs had an EC50 of approximately 1 nM); Figure 2 and Table 4.

Table 4: Binding affinities for cellular FAP

Antibodies	TOP (MFI)	EC ₅₀ (nM)
ab1z12-hFc	16376	0.736
ab3z7-hFc	12644	0.843
ab3z10-hFc	12704	0.777
4B9 -hFc	15350	0.718
Sibrotuzumab	20635	1.118
IgG1 isotype control	NA	NA
ab1-hFc	15938	0.375

10 The results demonstrate that the antibody constructs specifically bind cFAP but do not bind sFAP and, thus, are selective for and distinguish between the two antigens. In contrast, the known control antibody sibrotuzumab and the antibody construct comprising the variable domain of the known antibody 4B9 bind both sFAP and cFAP.

15 **6.4 Example 3 - Competitive flowcytometry showing preferential binding to cellular FAP**

A competitive flowcytometry assay was performed to further verify the selective binding of the constructs of Example 2 for cFAP over spFAP. Specifically, the flowcytometry assay of Example 2 was repeated wherein the cFAP expressing cells were incubated with the antigen-binding constructs in the presence or absence of human plasma. Human plasma is known to contain spFAP at a concentration of 100 ng/ml (median value from multiple studies); see, e.g. Busek et al., Frontiers in Bioscience 23(2018), 1933-1968.

In the competitive flow cytometry assay, sibrotuzumab and 4B9 were observed to exhibit a notable decrease in MFI towards cells with cFAP in the presence of human plasma (spFAP) as compared to assays in the absence of human plasma. Such a decline in binding in the presence of plasma (spFAP) reached 83% for sibrotuzumab and 95% for 4B9. This significant drop in MFI
5 underscores that soluble plasma FAP (spFAP) acts as a competitive sink, markedly affecting the potential therapeutic efficacy of known anti-FAP antibodies and derivatives thereof.

In contrast, the competitive flowcytometry assay showed preferential binding of ab3z7-hFc to cellular FAP (cFAP) in the presence of plasma FAP (spFAP). The minimal reduction of the
10 binding capacity of ab3 in the presence of plasma was similar to that of the isotype IgG, which was used as a negative control. The results further confirm that ab3z7 selectively bound to and was capable of distinguishing cFAP over spFAP. Figure 3

6.5 Example 4 – Flow cytometry demonstrating the cross-reactivity of cFAP specific and 15 selective antigen binding molecules to cFAP of other mammalian species

Flow cytometry was performed on HEK293F transfected transiently with cynomolgus or murine cFAP (see materials and methods, above). Testing of the antigen binding molecules showed that ab1-hFc, ab1z12-hFc, ab3-hFc, ab3z7-hFc and ab3z10-hFc, exhibited binding
20 activity to at least one of cynomolgus cFAP (Figure 4A) or murine cFAP (Figure 4B). Therefore, the antigen binding molecules provided and developed according to the invention are cross-reactive to at least one non-human mammal cFAP, which is beneficial for pre-clinical development.

25 6.6 Example 5 – PK profile

In a pharmacokinetic (PK) study, female BALB/c mice aged between 6 to 8 weeks received a single intravenous injection of ab1-hFc or 4B9-hFc at a dosage of 10 mg/kg. Blood samples were systematically collected and preserved post-administration over a period of 2 weeks.
30 The samples were batch analysed at the end of the experiment using human IgG1 ELISA to quantify the concentration of the test compound over time (Figure 5A).

The resulting data was used to calculate the PK parameters half-life, clearance rate, and area under the curve (AUC) to provide the constructs’ pharmacokinetic profile (Figure 5B and Table 5). Each data point was obtained from at least 3 mice.

5 Molecular weight is associated with the clearance rate, where larger molecular weight polypeptides have longer half-life and AUC. Prior art anti-FAP antibodies have been shown to have poor half-life due to their binding to spFAP (see, e.g. Kloft et al., Invest New Drugs 22(2004), 39-52; Waldhauer et al., MAb 13(2021), 1913971). As shown in Figure 5B and in Table 5, ab1-hFc (molecular weight of 80 kD) exhibited an improved AUC/Dose and
10 AUC/Dose/Mwt compared to control 4B9-hFc (formatted as a complete IgG1, Mwt 148KD). The parameters are calculated from the respective antibody concentration derived from a human IgG1 ELISA based on a standard curve.

Table 5: PK parameters

Parameter	Dose group		
	ab1-hFc	4B9-hFc	Units
Dose	10	18.5	mg/Kg
Molecular weight	80	146	KD
Dose / Molecular Weight	0.13	0.13	mg/Kg/Mwt
AUC _{0-infinity} /Dose	933	822	h*kg*µg/mL/mg
AUC _{0-infinity} /Dose/MWt	11.96	5.63	h*kg*µg/mL/mg/Mwt

15 These results show that the antigen binding molecules/constructs exhibited improved PK characteristics not only due to their low molecular weight, but also due to their selective binding to cFAP over spFAP in the plasma.

20 **6.7 Example 6 – Impact of specific and selective cFAP antigen binding molecules on spFAP levels in an *in vivo* murine model**

The activity of specific and selective cFAP antigen binding molecules on the levels of circulating spFAP was assessed in an *in vivo* mouse model. Female Balb/C mice were injected
25 intravenously three times per week over a period of two weeks with vehicle only, a cFAP selective antigen binding -Fc construct or 4B9-Fc (both Fc formats used murine IgG2a) at a

dose of 5 mg/kg. Animals were sacrificed and murine plasma samples collected at day 43, i.e. 2 weeks after the last administration of the antigen-binding constructs.

A murine sandwich ELISA (Mouse FAP DuoSet ELISA, Biotechne DY8647-05) was used to assess the levels of spFAP in the murine plasma samples (diluted 1:100 and 1:500 in dilution buffer). The cFAP-Fc antigen binding construct of the invention had a higher affinity to cFAP and a lower molecular weight as compared to 4B9-Fc. spFAP in plasma was depleted in mice that received 4B9-Fc while sFAP was preserved in mice that received the specific and selective cFAP-Fc construct (Figure 6). This demonstrates that the cFAP antigen binding construct does not interact with soluble FAP in plasma and maintains stable plasma FAP (spFAP) levels throughout the course of treatment and after treatment.

6.8 Example 7 – FAP enzymatic activity

It is recognized that FAP has important physiological functions, e.g. cleaving neuropeptides and extracellular matrix components. Therefore, it is important that the selective cFAP antigen binding molecules do not affect the enzymatic activity of FAP to avoid any unintended side-effects. It was demonstrated that the cFAP specific and selective antigen-binding molecules and constructs did not bind to spFAP in plasma and did not affect circulating spFAP plasma levels. To further confirm that the selective cFAP antigen binding constructs did not potentially negatively impact FAP function, FAP enzymatic activity was tested in the presence or absence of the selective cFAP constructs of the present invention or of control, known anti-FAP molecules.

The FAP enzyme activity assay (see, general Materials and Methods, above) demonstrated that in the absence or presence of ab1-hFc and ab3-hFc, the enzymatic activity of recombinant FAP (Figure 7A) and human plasma comprising soluble FAP (spFAP; Figure 7B) was unaffected at all tested concentrations. The specific and selective cFAP constructs/antibodies, sibrotuzumab, and 4B9-hFc did not affect the enzymatic activity of FAP. In contrast, it has previously been shown that anti-FAP antibodies can directly affect the enzymatic function of FAP (see, e.g. WO 2016/110598A1).

6.9 Example 8 – Antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) showed enhanced effector cell binding

The cFAP antigen-binding constructs specifically and selectively bind cFAP. The constructs as reported in Example 2 also were designed to enhance Fc receptor binding through known modification to the Fc domains, including afucosylation and/or the modifications within the Fc region (e.g. DEL mutation) as mentioned, e.g. in Lazar et al., PNAS 103(2006), 4005-4010. By containing modified Fc domains, the cFAP antigen binding constructs can engage Fc receptors on immune effector cells and mediate effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP).

The cytotoxic activity against primary human fibroblasts as target cells was demonstrated using the cFAP antigen binding constructs ab1-hFc and ab3-hFc. Increasing concentrations of ab1-hFc and ab3-hFc were incubated with fibroblasts and FcγRIIIa-expressing effector cells. The ADCC Reporter assay showed that the tested constructs efficiently induced ADCC in the tested FAP-expressing target cells uterine (HUF), cardiac (HCF), dermal (Detroit 551) and pulmonary (HPF) fibroblasts (Figure 8A-D, respectively). Sibrotuzumab was included in the assays against HCF and Detroit 551 cells for comparison.

Cytotoxic activity of the cFAP antigen binding constructs was also tested in an ADCP assay. Briefly, the FcγRIIIa ADCP Bioassay (Promega) used bioluminescent Jurkat T cells expressing FcγRIIIa and target cells HPF, HCF, HUF, or Detroit 551 expressing cFAP. The tested specific and selective cFAP antigen-binding constructs were ab3-hFc, ab1-hFc and one or both of ab1-hFc DEL (comprising the DEL mutation known to enhance effector functions, Ser239Asp, Ala303Leu and Ile332Glu according to EU numbering) or an afucosylated Fc region (antibodies with suffix -ahFc) as shown in Table 6. Sibrotuzumab was also tested as a reference.

Table 6: Antibody constructs with effector function enhancing modifications

Name	Sequence	SEQ ID NO:
ab1-hfc(DEL)	QVQLVESGGGLVQAGDSLSLSCVASAPTRSTYAMGWFRQAPGKEREF AAVINWSGTLTSYADSVKGRFTISRDNAKNTVFLQMNSLKPDDTAVYY CAADRFRTVGSRPSYWGQGPVTVSSGSEPKSSDKTHTCPPCPAPEL LGGPDPVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPL PEEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTTPVLDSGGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK	32
ab1-ahFc	QVQLVESGGGLVQAGDSLSLSCVASAPTRSTYAMGWFRQAPGKEREF AAVINWSGTLTSYADSVKGRFTISRDNAKNTVFLQMNSLKPDDTAVYY CAADRFRTVGSRPSYWGQGPVTVSSGSEPKSSDKTHTCPPCPAPEL LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTTPVLDSGGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK	33

The specific and selective cFAP constructs were incubated with ADCP effector cells and the FAP-expressing target cells, HUF (Figure 9A and 10A), HCF (Figure 9B) or HPF (Figure 10B), and. It was observed that the specific and selective cFAP constructs with enhanced FcγR binding activity showed ADCP activity.

Furthermore, the modified variants comprising the DEL mutation or an afucosylated Fc region were tested in the ADCC assay as described above. As shown in Figure 11, the effector function-enhancing modifications enhanced luminesce in the ADCC assay for each of the employed target cells lines: HUF (Figure 11A), HPF (Figure 11B), HCF (Figure 11C/D) and Detroit 551 (Figure 11E). Thus, it was shown that ADCP activity can be imparted to the specific cFAP constructs of the present invention and that the ADCC activity can be enhanced by modification of the Fc region.

6.10 Example 9 – Antigen binding molecules specific and selective for cFAP are not internalised on binding to cFAP

The specific and selective anti-cFAP molecules developed according to the methods provided herein did not show internalisation when incubated with WI-38 cells expressing cFAP. The internalisation rate was comparable to the Isotype control, 4B9-hfc, and Sibrotuzumab at 4h and 24h. The EC₅₀ for all antibodies was not calculable even though concentrations up to 200nM were used (4-fold serial dilutions, 8 concentrations).

6.11 Example 10 – Antigen binding molecules specific and selective for cFAP do not bind cFAP homologue DPP4

The specific and selective anti-cFAP molecules ab1z10-hFc, ab3z7-hFc, ab3z10-hFc did not show significant binding to HEK293F expressing cellular DPP4 up to 100nM. The binding to DPP4 of the aforementioned antigen binding molecules was similar to the Isotype control used, as well as Sibrotuzumab.

6.12 Example 11 – ADCP in human PBMC-derived macrophages was improved in ab3z7-hFc compared to Sibrotuzumab

The specific and selective anti-cFAP molecule ab3z7-hFc showed superior phagocytosis efficacy compared to Sibrotuzumab in terms of EC₅₀ (10-fold improvement) and maximal phagocytosis (44.86% improvement) (Figure 12).

Table 7: Results of the ADCP assay

Effector Cell	Target Cell	E:T	Antibody	EC ₅₀ (nM)	Max Phagocytosis (%)
Human PBMC Macrophage	HT1080-FAP	1:1	Afucosylated Isotype control	NA	5.73
			Ab3z7-ahFc	0.010	18.00
			Sibrotuzumab	0.014	14.20

6.13 Example 12 – ab3 conjugated to a fluorescent probe shows specific uptake in mice with tumor positive for human FAP

The specific and selective anti-cFAP molecule ab3 was conjugated to a fluorescent probe and showed specific staining in mice with a tumor positive for human FAP (Figure 13).

Table 7: Antibody construct used for *in-vivo* imaging

Name	Sequence	SEQ ID NO:
Ab3z7p2-Scarlett	QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISW SGGTTQYTDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAADRDFRTVG SRPSYWGGQTTVTVSSHHHHHHGGGSGGGGSMDS TEAVIKEFMRFKVHME GSMNGHEFEIEGEGEGRPYEGTQTAKLRVTKGGPLPFSWDILSPQFMYGSRA FTKHPADIPDYWKQSFPEGFKWERVMNFEDGGAVSVAQDTSLEDGTLIYKVK LRGTNFPDPGPVMQKKTMGWEASTERLYPEDVVLKGDIKMALRLKDGGRYL ADFKTTYRAKKPVQMPGAFNIDRKLDITSHNEDYTVVEQYERSVARHSTGGSG GS	35

6.14 Example 13 – ab3-mFc has low binding to all healthy human tissues

IHC staining using ab3-mFc shows low binding to all healthy adult tissues of both genders (Figure 14). In addition to the tissues in Figure 14, the following tissues had no visually detectable staining of human FAP by ab3-mFc: bone marrow, cerebellum, brain, pituitary, fallopian tube, kidney, ovary, prostate, spinal cord, thymus, thyroid, ureter, cervix.

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CLAIMS

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1. An antigen-binding molecule specific for membrane bound fibroblast activation protein (FAP) as expressed on the surface of a cell-membrane (cFAP), which antigen binding molecule discriminates between said cFAP and an extracellular soluble non-membrane associated fibroblast activation protein (sFAP).

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2. The antigen-binding molecule according to claim 1 wherein said cFAP has the amino acid sequence of SEQ ID NO:1 and is expressed on the surface of a HEK293 cell, and wherein said sFAP has an amino acid sequence of SEQ ID NO:2 or is as present in human plasma (spFAP).

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3. The antigen-binding molecule according to claim 1 or 2, wherein said molecule has an EC₅₀ for binding to cFAP of less than 5nM and an EC₅₀ for binding to sFAP that is at least 20 fold weaker.

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4. The antigen-binding molecule according to claim 3, wherein said EC₅₀ for binding to sFAP is determined as the EC₅₀ for binding to human plasma known or believed to contain spFAP.

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5. The antigen-binding molecule according to claim 3 or 4, wherein said molecule has the property (A) as follows

(A) the EC₅₀ of said molecule for binding to spFAP is not detectable or incalculable when assessed in an assay, in which assay, and where assessed under the same conditions, the EC₅₀ of sibrotuzumab or 4B9 for binding to said spFAP is <1nM or <10nM, respectively;

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and

said antigen-binding molecule has one or more of the following cFAP binding properties determined by flow cytometry:

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- (a) an apparent K_d of binding to said cell expressing said cFAP of less than 2nM;
- (b) an EC₅₀ of binding to said cell expressing said cFAP of less than 2 nM;
- (c) an EC₅₀ of binding to said cell expressing said cFAP that is within at least 1-fold of the EC₅₀ of sibrotuzumab or 4B9 assessed under the same conditions;

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- (d) a maximum binding to said cell expressing said cFAP that is within 1-fold of sibrotuzumab or 4B9 assessed under the same conditions;

and/or has the property (B) as follows

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- (B) the antigen-binding molecule according to any one of claims 3 to 5, wherein the ratio of binding to cFAP in the presence of undiluted human plasma having an spFAP concentration of at least 80ng/mL relative to the binding in the absence of said undiluted human plasma is at least 80% at an antigen-binding molecule concentration of 0.8nM, at least 60% at an antigen-binding molecule concentration of 0.16 nM, or at least 50% at an antigen-binding molecule concentration of 0.0032 nM; wherein as assessed in the same assay and under the same conditions,

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- (i) the ratio of binding for 4B9 to cFAP in the presence of said undiluted human plasma relative to the binding in the absence of said undiluted human plasma is less than 10% at a 4B9 concentration below 0.8nM; or
- (ii) the ratio of binding for sibrotuzumab to cFAP in the presence of said undiluted human plasma relative to the binding in the absence of said undiluted human plasma is less than 25% below 0.8nM.

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6. The antigen-binding molecule according to any one of claims 1 to 5, which molecule comprises or consists of an antibody, an antibody antigen-binding domain or an antibody antigen-binding fragment.

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7. The antigen-binding molecule according to claim 6, wherein said molecule comprises more than one antibody, antibody antigen-binding domain, or antibody antigen-binding fragment.

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8. The antigen-binding molecule according to claim 7 comprising more than one antibody antigen-binding domains or more than one antibody antigen-binding fragments, which more than one antibody antigen-binding domains or antibody antigen-binding fragments have the same amino acid sequence, or which more than one antibody antigen-binding domains or fragments specifically bind the same antigen and have different amino acid sequences.

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9. The antigen-binding molecule according to any one of claims 6 to 8 comprising at least one antibody antigen-binding domain or fragment, wherein said at least one domain or fragment is an Fab, Fab', F(ab')₂, Fv, scFv, or a single domain binding fragment.

10. The antigen-binding molecule according to claim 9, wherein said single domain binding fragment is a sdAb, dAb, nanobody or V_HH domain.
- 5 11. The antigen-binding molecule according to any one of claims 1 to 10, wherein said molecule comprises a human Fc domain.
- 10 12. The antigen-binding molecule according to claim 11, wherein said Fc domain (i) comprises the mutation S239D/A303L/I332E according to EU numbering (the DEL modification), (ii) is afucosylated, or (iii) is modified to increase ADCC or ADCP function relative to the same antigen-binding molecule comprising an IgG1 Fc domain having the amino acid sequence of SEQ ID NO:22.
- 15 13. The antigen-binding molecule according to any one of claims 7 to 12, wherein said antibody, antibody antigen-binding domain or antibody antigen-binding fragment is a human, chimeric, humanised, CDR-grafted, and/or deimmunised antibody, antibody antigen-binding domain or antibody antigen-binding fragment.
- 20 14. The antigen-binding molecule according to any one of claims 1 to 13, wherein said molecule comprises or consists of one or more V_HH domains.
- 25 15. The antigen-binding molecule according to claim 14, wherein said one or more V_HH domains comprise
- (i) a CDR1 having the sequence of X₁X₂TX₃X₄X₅YAX₆G (SEQ ID NO:3), wherein X₁ is A, G or S; wherein X₂ is P, R or G; wherein X₃ is K, F, R or N; wherein X₄ is S, R or G; wherein X₅ is T, S or N; and wherein X₆ is M, I or L;
- (ii) a CDR2 having the sequence of X₁IX₂WX₃X₄X₅X₆TX₇YX₈DSVKG (SEQ ID NO:4), wherein X₁ is A or V; wherein X₂ is N, W or S; wherein X₃ is S or A; wherein X₄ is G or N; wherein X₅ is T or G; wherein X₆ is I, L, S, T or V; wherein X₇ is S, Q or N; and wherein X₈ is S or T; and/or
- 30 (iii) a CDR3 having the sequence of AADX₁DFRTVGSRPSY (SEQ ID NO:5), wherein X₁ is R, S or K;
- and wherein said CDRs are defined according to IMGT numbering.
- 35 16. The antigen-binding molecule according to claim 14 or 15, wherein said one or more V_HH domain comprises
- (i) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7);

- (ii) a CDR2 having the sequence of VINWSGTLTSYADSVKG (SEQ ID NO:8), VINWAGTLTSYADSVKG (SEQ ID NO:9), VISWSGTLTSYADSVKG (SEQ ID NO:10), AISWSGGTTQYTD SVKG (SEQ ID NO:11), AISWSGGTTNYTD SVKG (SEQ ID NO:12), or AISWSGGTTNYADSVKG (SEQ ID NO:13); and/or
- 5 (iii) a CDR 3having the sequence of AADRDFRTVGS RPSY (SEQ ID NO:14).
and wherein said CDRs are defined according to IMGT numbering.
17. The antigen-binding molecule according to any one of claims 14 to 16, wherein said one or more V_HH domains comprise
- 10 (i) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VINWSGTLTSYADSVKG (SEQ ID NO:8) and a CDR3 having the sequence of AADRDFRTVGS RPSY (SEQ ID NO:14);
- (ii) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VINWAGTLTSYADSVKG (SEQ ID NO:9) and a CDR3 having the sequence of AADRDFRTVGS RPSY (SEQ ID NO:14);
- 15 (iii) a CDR1 having the sequence of APTRSTYAMG (SEQ ID NO:6) or GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of VISWSGTLTSYADSVKG (SEQ ID NO:10) and a CDR3 having the sequence of AADRDFRTVGS RPSY (SEQ ID NO:14);
- (iv) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTQYTD SVKG (SEQ ID NO:11) and a CDR3 having the sequence of AADRDFRTVGS RPSY (SEQ ID NO:14);
- 20 (v) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTNYTD SVKG (SEQ ID NO:12) and a CDR3 having the sequence of AADRDFRTVGS RPSY (SEQ ID NO:14) ; or
- 25 (vi) a CDR1 having the sequence of GRTFSSYAIG (SEQ ID NO:7), a CDR2 having the sequence of AISWSGGTTNYADSVKG (SEQ ID NO:13) and a CDR3 having the sequence of AADRDFRTVGS RPSY (SEQ ID NO:14) .
18. The antigen-biding molecule according to any one of claims 14 to 17, wherein said one or more V_HH domains comprise the sequence of
- 30 (i) QVQLVESGGGLVQAGDSLSCVASAPTRSTYAMGWFRQAPGKEREF AAVINWSGTLTSYADSVKG RFTISRDN AKNTVFLQMNSLKPDDTAVYYCAADRDFRTVGS RPSYWGQ GTPVTVSS (SEQ ID NO:15);

- (ii) QVQLVESGGGLVEPGDSLRLSCAASGRTFSSYAIGWFRQAPGKEREFVAAISWSGGTT
NYTDSVKGRFTISRDNANTVYLQMNSLKPDDTAVYYCAADRDFRTVGSRPSYWSKGT
RVTVSS (SEQ ID NO:16);
- (iii) QVQLVESGGGLVQPGGSLRLSCSASAPTRSTYAMGWFRQAPGKEREFASVISWSGTLT
SYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGQGT
TVTVSS (SEQ ID NO:17);
- (iv) QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISWSGGTT
QYTDSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAADRDFRTVGSRPSYWGQGT
TVTVSS (SEQ ID NO:18);
- (v) QVQLVESGGGLVQPGGSLRLSCSASGRTFSSYAIGWFRQAPGKEREFVSAISWSGGTTN
YADSVKGRFTISRDNKNTLYLQMNSLRPEDTAVYYCAADRDFRTVGSRPSYWGQGT
VTVSS (SEQ ID NO:19); or
- (vi) a variant amino acid sequence having at least 85%, 90% or 95% sequence
identity to any one of SEQ ID NOs:15 to 19, wherein said variant is the
modification of any one of SEQ ID NOs:15 to 19 by one or more conservative
amino acid substitutions..
19. The antigen-binding molecule according to any one of claims 1 to 18 for use as a
medicament.
20. The antigen-binding molecule according to any one of claims 1 to 18 for use in the
treatment of a disease characterized by the overexpression of cFAP, preferably
wherein said treatment is for use in a subject having a blood spFAP concentration of
greater than 40 ng/ml.
21. The antigen-binding molecule according to any one of claims 1 to 18 which is a
diagnostic for use *in vitro* or *in vivo*
- (i) for the detection of cFAP; and/or
- (ii) for discriminating cFAP from spFAP in serum, plasma, tissue or bodily fluids.