

IMPROVED BISPECIFIC ANTI-TUMOR ANTIGEN/ANTI-HSG ANTIBODIES FOR PRE-TARGETING OF HYPERPROLIFERATIVE DISORDERS

FIELD OF THE INVENTION

The invention refers to a bispecific anti-tumor antigen/anti-HSG antibody with
5 selected light and heavy chain variable domains which allows improved pre-targeting of
malignancies, specifically of tumors, more specifically of solid tumors. The humanized
anti-HSG (histamine-succinyl-glycyl) binding site binds with high affinity to molecules
containing the moiety histamine-succinyl-glycyl (HSG). Methods of making the bispecific
anti-tumor antigen/anti-HSG comprising humanized anti-HSG variable domains are also
10 disclosed. The bispecific antibody can further have properties such as reduced
aggregation potential and reduced hydrophobicity due to selected amino acid
substitutions in the variable domains. The anti-tumor antigen/anti-HSG antibodies can
be used for diagnosis and treatment of subjects suffering from cancer.

BACKGROUND OF THE INVENTION

15 Tumor targeting with monoclonal antibodies is an attractive approach for selective
cancer therapy. Radiolabelled antibodies have proven effective in patients with
haematological malignancies, but adaptation of radioimmunotherapy (RIT) in solid
tumors has been challenging. The slow blood clearance and delayed tumor uptake of
directly radiolabelled antibodies cause continuous radiation exposure to normal organs
20 and the bone marrow and are producing high background signal when used as
diagnostic tools. Pre-targeting techniques were developed to overcome these
limitations. With pre-targeting, a non-radiolabelled humanised bispecific monoclonal
antibody (bsMAb) is administered first intravenously. After the bsMAb localises in the
tumor and clears from the circulation, a radiolabelled hapten peptide is given that is
25 rapidly trapped in the tumor by the bsMAb, while the remainder clears from the blood
very quickly, being eliminated via the kidneys (Stawski R.S. et al., 2015). Pre-targeting
reduces the radiation exposure to radiosensitive normal tissues, such as bone marrow,
as well as other tissues (Stawski R.S. et al., 2015).

A tri-Fab bispecific monoclonal antibody TF2 divalent for the carcinoembryonic
30 antigen (CEACAM-5, or CEA) and monovalent for histamine-succinyl-glycine (HSG)
hapten peptide was described in Schoffelen R. et al., 2013, and Rossi E.A. et al., 2006.
TF2 binds to the tumor-associated antigen CEACAM-5 expressed on the cell surface of

colon tumors. Subsequently, a HSG hapten peptide IMP288 carrying a radionuclide is administered, binding to the anti-HSG arm on the bispecific mAb (Schoffelen R. et al., 2013). Phase I study NCT00860860 where, depending on the characteristics of the radionuclide used (^{111}In , or ^{177}Lu), CEACAM-5-expressing tumor cells were radioimaged, and/or treated radioimmunotherapeutically, demonstrated for the first time that pre-targeting with TF2 and radiolabelled IMP288 in patients with CEACAM-5-expressing colorectal cancers is feasible and safe. With this pre-targeting method, tumors were specifically and rapidly targeted (Schoffelen R. et al., 2013).

The anti-HSG mAb sequences in the TF2 are from the humanized anti-HSG mAb clone 679 that was originally identified from mouse hybridoma (Morel A., et al., 1990). The mouse anti-HSG mAb 679 and the humanized mAb 679 are described in US20090246131 and US20090240037A1, respectively. In US 2009/0240037 A1, the humanized Ab 679 was generated and tested in the single chain variable fragment (scFv) format as anti-CEACAM-5 x anti-HSG diabody carrying the C-terminal 6xHis tag. However, the humanized HSG mAb of the art still has several disadvantages. The mAb is still very close to mouse amino acid sequence yet leading to unwanted immunogenicity of said antibodies. Furthermore, the production of the Tri-Fab (anti-tumor antigen x anti-HSG) antibody requires the use of special technique known as dock-and-lock method (DNL). DNL involves the use of a pair of distinct protein domains involved in the natural association between cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and A-kinase anchoring proteins (AKAPs). The dimerization and docking domain found in the regulatory subunit of PKA and the anchoring domain (AD) of an interactive AKAP are each attached to a biologic entity, and the resulting derivatives, when combined, readily form a stably tethered complex of a defined composition. Attaching further non-antibody subunits to the Fab arms, additionally adds risk of immunogenicity of said antibodies. Furthermore, the large-scale manufacturing of DNL Tri-Fabs is considered very challenging.

The cytokine Macrophage Migration Inhibitory Factor (MIF) has been described as early as 1966 (David, J.R., 1966; Bloom B.R. and Bennet, B., 1966). The biochemical properties and physiological role of MIF were elucidated following its cloning and recombinant expression (Bernhagen et al., 1993; Bernhagen et al., 1994). It is now well accepted that MIF is a pivotal regulator of innate immunity, playing a central role in inflammatory responses and cancer.

MIF has been shown to be up-regulated in a large variety of human neoplasms like pancreatic, breast, prostate, colon, brain, skin, and lung-derived tumors (Bando et al., 2002; Chen et al., 2010; Kamimura et al., 2000; Meyer-Siegler and Hudson, 1996; Shimizu et al., 1999; Takahashi et al., 1998; Winner et al., 2007). Several studies report
5 that MIF expression closely correlates with tumor aggressiveness and metastatic potential, suggesting that MIF may play an important role in disease severity and cell survival (Rendon et al., 2009). Recent data suggest that extracellular MIF may contribute to a more aggressive tumor phenotype as compared to intracellular MIF (Verjans et al., 2009). MIF contributes to a microenvironment that favors tumor growth, angiogenesis,
10 invasiveness, and metastasis. Besides its pro-inflammatory functions, MIF exerts anti-apoptotic and pro-proliferative effects, including inhibition of p53 (Hudson et al., 1999; Mitchell and Bucala, 2000) and activation of the central kinases ERK1/2 (Mitchell et al., 1999) and AKT (Lue et al., 2007). MIF further has been described as a pro-angiogenic factor promoting neo-angiogenesis (Coleman et al., 2008) and tumor vascularization by
15 stabilizing HIF-1 α (Winner et al., 2007) and up-regulation of pro-angiogenic factors like VEGF and IL-8 (Ren et al., 2004). MIF also acts as a chemokine and is expected to contribute to the inflammatory cell recruitment within the tumor environment via the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al., 2007; Rendon et al., 2007).

MIF, however, is markedly different from other cytokines and chemokines
20 because it is constitutively expressed and present in the circulation of healthy subjects. Pre-formed MIF is stored in cytoplasmic pools of macrophages, T-cells, and many other cells within the body, including the hypothalamic-pituitary-adrenal axis, allowing for rapid release upon stimulation without de novo synthesis (Bernhagen et al., 1993; Bacher et al., 1997; Fingerle-Rowson et al., 2003).

25 Due to the ubiquitous nature of this protein, MIF can be considered as an inappropriate target for therapeutic intervention. However, MIF occurs in two immunologically distinct conformational isoforms, termed reduced MIF (redMIF) and oxidized MIF (oxMIF) (Thiele M. et al., 2015). RedMIF was found to be the abundantly expressed isoform of MIF that can be found in the cytoplasm and in the circulation of
30 any subject. RedMIF seems to represent a latent non-active storage form (Schinagl. A. et al., 2018).

In contrast, oxMIF seems to be the physiologic relevant and disease related isoform which can be detected in tumor tissue, specifically in tumor tissue from patients with colorectal, pancreatic, ovarian and lung cancer outlining a high tumor specificity of

oxMIF (Schinagl. A. et al., 2016), but also in the circulation and in the inflamed tissue of patients with inflammatory diseases (Thiele et al., 2015).

Antibodies targeting oxMIF showed efficacy in *in vitro*- and *in vivo* models of inflammation and cancer (Hussain F. et al., 2013; Schinagl. A. et al., 2016; Thiele et al.,
5 2015).

WO2019/234241A1 discloses anti-oxMIF/anti-CD3 bispecific antibodies.

WO2009/086920A1 describes the anti-oxMIF antibody Bax69 (Imalumab).

WO2022069712A1 describes anti-oxMIF antibodies with reduced aggregation potential and reduced hydrophobicity.

10 Protein aggregation, specifically antibody aggregation is frequently observed at several stages of bioprocessing, including protein expression, purification, and storage. Antibody aggregation can affect the overall yield of therapeutic protein manufacturing processes and may contribute to stability and immunogenicity of therapeutic antibodies.

Protein aggregation of antibodies thus continues to be a significant problem in
15 their developability and remains a major area of focus in antibody production. Antibody aggregation can be triggered by partial unfolding of its domains, leading to monomer-monomer association followed by nucleation and aggregate growth. Although the aggregation propensities of antibodies and antibody-based proteins can be affected by the external experimental conditions, they are strongly dependent on the intrinsic
20 antibody properties as determined by their sequences and structures.

For example, resistance to aggregation can be achieved by stabilizing the native state (i.e., resisting unfolding) or by reducing the propensity of the unfolded or partially folded states of the protein to aggregate. A disadvantage of stabilizing the native state is that proteins will likely be exposed to an environment in which they will unfold.
25 Generally, when a protein is denatured or unfolds, amino acid residues that normally mediate intramolecular contacts in the interior of the protein are exposed. Such exposure often makes proteins prone to form intermolecular contacts and aggregate. In contrast to proteins that resist unfolding, a protein having a reduced propensity to aggregate when unfolded will simply refold into a bioactive non-aggregated state after exposure to
30 such an environment.

The aggregation-resistance or aggregation-propensity of antibodies and proteins comprising antigen binding domains thereof is usually limited by the most aggregation prone domain(s) contained therein and by the strength of its interaction with surrounding domains (if present).

This is because once that domain unfolds, if it is incapable of refolding, it may interact with other domains in the same protein or in other proteins and form aggregates. Constant domains of antibodies generally do not aggregate and do not vary considerably. Accordingly, the weakest domains of an antibody with regards to aggregation potential and stability are generally considered to be the variable domains (e.g., heavy chain variable domain (V_H) and/or light chain variable domain (V_L), Ewert S. et al., 2003). In this regard, incorporation of aggregation prone V_H or V_L domains into otherwise stable recombinant antibody products often imparts these generally undesirable traits to the new recombinant design. Thus, engineering a variable domain to be aggregation-resistant is most likely to render the entire protein which may comprise that variable domain aggregation-resistant. Various strategies have been proposed for reducing aggregation of variable domains, e.g., rational design of aggregation-resistant proteins, complementarity determining region (CDR) grafting, or introducing disulfide bonds into a variable domain. Rational design of aggregation-resistant proteins generally involves using in silico analysis to predict the effect of a point mutation on the aggregation propensity of a protein, however, a pure in silico prediction does not necessarily lead to an actual improvement in this respect.

Reduction in aggregation propensity has been shown to be accompanied by an increase in expression titer, showing that reducing protein aggregation is beneficial throughout the development process and can lead to a more efficient path to clinical studies. For therapeutic proteins, aggregates are a significant risk factor for deleterious immune responses in patients and can form via a variety of mechanisms. Controlling aggregation can improve protein stability, manufacturability, attrition rates, safety, formulation, titers, immunogenicity, and solubility (Wei Li et al., 2016; Van der Kant R. et al., 2017).

Modulating antibody effector functions to suit specific therapeutic purposes is also of growing interest. Specifically, Fc-null or Fc-silenced antibodies may be a strategy to abrogate Fc effector function as strong immune effector functions through FcγR and complement interactions may sometimes be detrimental to antibody mechanisms.

WO2023/031397A1 describes Fc-silenced anti-oxMIF antibodies comprising amino acid substitutions in the light and heavy chain variable domains.

Schinagl et al., published April 1, 2023, report pre-targeted immunotherapy with anti-oxMIF/HSG bispecific antibody.

Fc gamma receptors (FcγRs) are a well described family of proteins including membrane-bound surface receptors, atypical intracellular receptors, and cytoplasmic glycoproteins. FcγRs control the humoral and innate immunity, which are essential for appropriate responses to infections and prevention of chronic inflammation or autoimmune diseases. Membrane-bound receptors are, for example, FcγRIIa, FcγRIIb-, FcγRIIIa, and FcγRIa receptors. Antibodies can regulate immune responses through interacting with the FcγRs.

On innate immune effector cells, activating and inhibitory FcγRs set a threshold for cell activation by immune complexes. Important examples for effector responses that are regulated by FcγRs are phagocytosis, ADCC and the release of inflammatory mediators. On dendritic cells (DCs), paired FcγR expression regulates cell maturation and antigen presentation, thereby indirectly controlling the cellular immune response. On B cells, the inhibitory FcγRIIB is essential for the maintenance of humoral tolerance. It acts as a late checkpoint at the level of class-switched memory B cells, plasmablasts or plasma cells. In addition, FcγRIIB has an important role in regulating plasma-cell homeostasis and survival. The antibody–FcγR interaction is influenced by several factors that have an impact on the expression level of activating and inhibitory FcγRs (such as cytokines) or change the affinity of the antibody–FcγR interaction (such as differential antibody glycosylation). Depending on the specific glycosylation pattern, IgG molecules can have enhanced pro- or anti-inflammatory activities. Importantly, antibody glycosylation is regulated during immune responses.

Atypical FcγRs are neonatal Fc receptor (FcRn) and cytoplasmic glycoproteins such as complement factor C1q protein. FcRn is expressed by endothelial cells, which internalize serum components including soluble IgGs from the bloodstream by pinocytosis. IgG binding to FcRn is pH dependent; the acidic pH (pH 6.0) inside the endosomal compartment allows the IgGs to bind to FcRn. After recycling back to the cell surface, the IgG dissociates from FcRn at physiological pH (~pH 7.2), is released back into the blood circulation and thereby protected from lysosomal degradation, leading to prolonged half-life of IgGs. FcRn therefore functions as the recycling of transcytosis receptor that is responsible for maintaining IgG and albumin in the circulation. Modifications of the Fc region resulting in decreased or silenced Fc regarding FcRn binding are known in the art and are described e.g. in Kenanova V. et al., 2005 and Pyzik M. et al., 2019.

Likewise, modulating the neonatal Fc receptor (FcRn) binding of IgG antibodies in order to modulate pharmacokinetics has also gained increased notoriety.

Specifically in view of above, the comprehensive adaptation of antibodies to the respective areas of application is and remains a challenge. Therefore, a need still exists
5 in the field for improved bispecific antibodies, especially for use in more effective targeted delivery methods, specifically pre-targeting, for therapeutic and diagnostic agents.

SUMMARY OF THE INVENTION

It is the objective of the present invention to provide improved bispecific
10 antibodies for pre-targeting of tumors for subsequent specific delivery of therapeutic or diagnostic agents.

The objective is solved by the subject matter of the present invention.

The inventive antibodies bind selectively to tumor antigens and HSG hapten moieties. Said antibodies have significant affinity for the tumor antigen and sufficient
15 residence time at the desired tumor location. Non-tumor-antigen bound antibodies are cleared from the circulation and exposure of normal tissues by subsequent administered therapeutic or diagnostic agents is minimized.

Herein provided are bispecific anti-tumor antigen/anti-HSG antibodies, comprising a binding site specifically recognizing a tumor antigen and a binding site
20 specifically recognizing HSG, comprising

- a light chain variable (VL) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 17, 19, 89, 90, and 91, or
- a light chain variable (VL) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 17, 19, 89, 90, and 91 with one or two further amino
25 acid substitutions and with glutamine at position 100 (Q100), glutamic acid at position 105 (E105), and isoleucine at position 106 (I106) according to Kabat numbering, and
- a heavy chain variable (VH) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10, or
- a heavy chain variable (VH) domain comprising a sequence selected from the
30 group consisting of SEQ ID NOs: 4, 6, 8, and 10 with one or two further amino acid substitutions and with arginine at position 19 (R19) according to Kabat numbering.

The bispecific anti-tumor antigen/anti-HSG antibody of the invention has very low immunogenicity, specifically due to the modified anti-HSG VL and VH domains. The

inventive antibody further has increased expression titers, high monomeric purity, and less cleavage of the anti-HSG heavy chain, thus making them highly feasible for manufacturing.

5 The antibody of the present invention has a highly selective binding specificity to the tumor antigen and the HSG hapten, and shows high storage stability. Targeted antibody modification can reduce FcRn binding and thus can optimize the half-life of the inventive antibody specifically when used in pre-targeting.

According to a specific embodiment, the antibody of the invention is of a Fab-scFv-Fc format.

10 According to a specific embodiment of the invention, the antibody described herein comprises a single-chain variable fragment (scFv) specifically recognizing HSG and is of the formula selected from the group consisting of VH-linker-VL-linker, VL-linker-VH-linker, wherein the linker comprises SEQ ID NO: 39 or SEQ ID NO: 42.

15 According to an alternative specific embodiment, the antibody has a CrossMab (CH1-CL crossover) format. i.e. a Fab carrying a CH1-CL domain crossover.

Specifically, the antibody of the invention comprises VL and VH domains specifically recognizing HSG, wherein the VL domain is C-terminally connected to a CH1-cross comprising SEQ ID NO: 30, and the VH domain is C-terminally connected to a CL-cross comprising SEQ ID NO: 38.

20 In a further embodiment, the antibody described herein comprises a Fc region comprising SEQ ID NO: 31, or a sequence having at least 95%, specifically 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 31, comprising asymmetric mutations in each CH3 domain enabling heterodimerization of two CHs from different antibodies, specifically one CH comprises „knob“ mutations T366W and S354C and one CH
25 comprises „hole“ mutations T366S, L368A, Y407V and Y349C, according to EU numbering index.

Specifically, the inventive antibody comprises a variant Fc region with reduced or eliminated effector functions and/or FcRn binding.

30 Specifically, the inventive antibody has a variant Fc region having amino acid substitutions at any one or more of positions E233, L234, L235, G236, G237, P238, I253, D265, S267, H268, N297, S298, T299, H310, E318, L328, P329, A330, P331, H435 of SEQ ID 31 according to EU numbering index, and optionally an aglycosylated Fc region.

Specifically, the antibody of the present invention contains two constant heavy chain domains having a hinge and a Fc region comprising SEQ ID NO: 35 and SEQ ID NO: 32.

In an alternative embodiment, the antibody of the present invention contains two
5 constant heavy chain regions having a hinge and a Fc domain comprising SEQ ID NO: 36 and one of SEQ ID NO: 33 or SEQ ID NO: 34.

In a further embodiment of the invention, the anti-tumor antigen binding of the antibody described herein is directed to oxMIF, Mesothelin (MSLN), and Folate Receptor alpha (FR α), specifically the antibody binds to oxMIF.

10 In a specific embodiment of the invention, the binding site specifically recognizing oxMIF comprises,

- a light chain variable domain comprising SEQ ID NO: 27, or a light chain variable domain comprising SEQ ID NO: 27 further comprising amino acid substitutions M30L and/or P80S, and

15 - a heavy chain variable domain comprising SEQ ID NO: 46, specifically with amino acid substitutions L5Q and/or W97Y,

wherein the amino acid positions are numbered according to Kabat.

According to a specific embodiment, the antibody described herein is selected from the group consisting of Fab-scFv-Fc, CrossMab, (scFv)₂-Fc, scFv/scFv-Fc,
20 Fab/(scFv)₂-Fc, Fab/Fab-scFv-Fc (IgG-central scFv), Fab/Fab-crossFab-Fc, Fab/crossFab-Fc, IgG-scFv, IgG-(scFv)₂.

In a further embodiment, the antibody is for use in the treatment or detection of malignancies, wherein said antibody is administered to a subject in a first step and a HSG moiety is administered in a second step, wherein said HSG moiety binds to the
25 antibody.

More specifically, said HSG moiety is conjugated to or labeled with one or more diagnostic and/or therapeutic agents, even more specifically said HSG moiety comprises one or more HSG haptens, one or more diagnostic and/or therapeutic agents, and a chelator.

30 In a further embodiment, the antibody described herein is bound to a HSG moiety conjugated to or labeled with one or more diagnostic and/or therapeutic agents, specifically said HSG moiety comprises one or more HSG haptens, one or more diagnostic and/or therapeutic agents, and a chelator.

Specifically, the chelator, which is conjugated to said HSG moiety, binds a radionuclide and is specifically selected from the group consisting of DOTA, DTPA, deferoxamine B (DFO) and DFO*.

Specifically, the radionuclide is selected from the group consisting of ^{67}Ga , ^{89}Zr ,
5 ^{111}In , ^{124}I , ^{131}I , ^{177}Lu , and ^{225}Ac .

In a further embodiment of the invention, the therapeutic agent is a radionuclide (radioisotope), or a cytotoxic agent, and the diagnostic agent is a radionuclide.

Further provided herein is the antibody for use in the preparation of a medicament.

10 Also provided herein is a pharmaceutical composition comprising the antibody together with a pharmaceutical excipient.

Specifically, the pharmaceutical composition provided herein is formulated for intravenous administration.

In a further embodiment of the invention, the pharmaceutical composition is for
15 use in the treatment of a patient suffering from cancer, specifically the treatment of tumors, solid tumors, more specifically the treatment of colorectal cancer, ovarian cancer, breast cancer, prostate cancer, pancreas cancer, and lung cancer.

Also provided herein is an isolated nucleic acid encoding the antibody described herein.

20 Provided herein is also an expression vector comprising the nucleic acid.

In a further embodiment of the invention, herein provided is a method for in vivo diagnosing cancer in a subject, wherein the antibodies described herein are used for detecting tumor cells.

In a further embodiment of the invention, herein provided is also a method for in
25 vitro diagnosing cancer, wherein the antibodies described herein are used for detecting tumor cells in a sample.

Herein provided is also a method for treating cancer using the antibodies described herein or the pharmaceutical composition described herein.

FIGURES

30 **Figure 1:** Schematic drawing of anti-Target X x anti-HSG bispecific mAbs having Fab-scFv-Fc, CrossMab (CH1-CL crossover), and IgG-central scFv formats. Left: Fab-scFv-Fc, central: CrossMab (CH1-CL crossover), right: IgG-central scFv.

Figure 2: Assessment of mAb C0132 and C0176-C0180 purity and severity of cleavage of anti-HSG scFv-Fc heavy chain by SDS-PAGE and Coomassie staining. A total of 3 µg of Protein A purified mAbs were resolved by NuPAGE™ 4-12% SDS-PAGE under reducing conditions ("red."). Spectra Multicolor Broad Range Protein Ladder was used as standard. Arrows show the anti-oxMIF LC and the cleaved anti-HSG heavy chain.

Figure 3: Deconvoluted mass spectrum of C0132 mAb. The 2 main peaks correspond to the intact antibody (126882.3 Da) and to the antibody with the truncated anti-HSG scFv-Fc heavy chain (100701.9 Da).

Figure 4: Stability of newly humanized anti-oxMIF x anti-HSG bispecific Fab-scFv-Fc mAbs compared to C0132 mAbs comprising previously humanized anti-HSG sequences. Stability was assessed by SEC before (day 0) and after storage for 83 days at -80°C (A) or 4°C (B). To allow direct comparison of the samples, values for % monomer were normalized to day 0 (= 100%) for each antibody.

Figure 5: Binding of the bispecific anti-oxMIF x anti-HSG mAbs to immobilized HSG. OD values at 450 nm (mean ± SEM, n=3) were plotted against mAb concentrations, and curve fitting was done by 4-parameter logistic fit using GraphPad Prism. (A) binding curve for Fab-scFv-Fc BsMAbs C0132, C0176, C0181, C0182, C0186, C0192 to HSG; (B) EC₅₀ values (mean ± SEM, n=3) of A; (C) EC₅₀ values (mean ± SEM, n=3) for binding of CrossMabs (C0255, C0238, C0239, C0240, C0241, C0245, C0250) to HSG. Dotted line represents the EC₅₀ value for of reference bispecific anti-oxMIF x anti-HSG antibodies.

Figure 6: Preserved binding of anti-oxMIF x anti-HSG bispecific mAbs towards immobilized MIF (oxMIF). Anti-oxMIF x anti-HSG Fab-scFv-Fc mAbs (A, B) and anti-CrossMabs (C) were bound to immobilized oxMIF and were detected by goat anti-human-IgG (Fc-specific)-HRP conjugate and binding curves for Fab-scFv-Fc bsAbs (A) and EC₅₀ values (mean ± SEM, n=2-3; B-C) are shown. C0008 (imalumab) was used as reference anti-oxMIF mAb.

Figure 7: Evaluation of off-target binding of anti-oxMIF x anti-HSG bsMAbs to A2780 MIF knock out cells. A2780 MIF^{-/-} cells were stained with serial dilutions of anti-oxMIF x anti-HSG bispecific mAbs C0176, C0181, C0182, C0186, C0192, and control therapeutic mAb rituximab. Binding was detected with AF488-conjugated goat anti-human IgG (H+L) secondary antibody on viable cells. Geometric mean fluorescence

intensity (MFI) values for AF488 channel were plotted against mAb concentrations in GraphPad Prism. Dotted line represents the staining (MFI) of secondary antibody only.

Figure 8: Thermal stability of newly humanized anti-oxMIF x anti-HSG CrossMabs. The thermal stability of the newly humanized anti-oxMIF x anti-HSG CrossMabs C0238, C0239, C0240, C0241, C0245, C0250, and the CrossMab C0255 having the sequences of the previously humanized anti-HSG Ab 679 was assessed by nanoscale Differential Scanning Fluorimetry (nanoDSF). The ratio of the monitored emission intensities (350 nm/330 nm) was plotted as a function of temperature, its first derivative was calculated and the temperature of the inflection point (T_{IP} , °C, mean $\pm$ SD, n= 4) of the first unfolding transition determined.

DETAILED DESCRIPTION

Unless indicated or defined otherwise, all terms used herein have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks, such as Sambrook et al, "Molecular Cloning: A Laboratory Manual" (4th Ed.), Vols. 1 -3, Cold Spring Harbor Laboratory Press (2012); Krebs et al., "Lewin's Genes XI", Jones & Bartlett Learning, (2017), and Murphy & Weaver, "Janeway's Immunobiology" (9th Ed., or more recent editions), Taylor & Francis Inc, 2017.

The subject matter of the claims specifically refers to artificial products or methods employing or producing such artificial products, which may be variants of native (wild-type) products. Though there can be a certain degree of sequence identity to the native structure, it is well understood that the materials, methods and uses of the invention, e.g., specifically referring to isolated nucleic acid sequences, amino acid sequences, fusion constructs, expression constructs, transformed host cells and modified proteins, are "man-made" or synthetic, and are therefore not considered as a result of "laws of nature".

The terms "comprise", "contain", "have" and "include" as used herein can be used synonymously and shall be understood as an open definition, allowing further members or parts or elements. "Consisting" is considered as a closest definition without further elements of the consisting definition feature. Thus "comprising" is broader and contains the "consisting" definition.

The term "about" as used herein refers to the same value or a value differing by $\pm 5\%$ of the given value.

As used herein and in the claims, the singular form, for example “a”, “an” and “the” includes the plural, unless the context clearly dictates otherwise.

As used herein, amino acids refer to twenty naturally occurring amino acids encoded by sixty-one triplet codons. These 20 amino acids can be split into those that
5 have neutral charges, positive charges, and negative charges:

The “neutral” amino acids are shown below along with their respective three-letter and single-letter code and polarity: Alanine (Ala, A; nonpolar, neutral), Asparagine (Asn, N; polar, neutral), Cysteine (Cys, C; nonpolar, neutral), Glutamine (Gln, Q; polar, neutral), Glycine (Gly, G; nonpolar, neutral), Isoleucine (Ile, I; nonpolar, neutral), Leucine
10 (Leu, L; nonpolar, neutral), Methionine (Met, M; nonpolar, neutral), Phenylalanine (Phe, F; nonpolar, neutral), Proline (Pro, P; nonpolar, neutral), Serine (Ser, S; polar, neutral), Threonine (Thr, T; polar, neutral), Tryptophan (Trp, W; nonpolar, neutral), Tyrosine (Tyr, Y; polar, neutral), Valine (Val, V; nonpolar, neutral), and Histidine (His, H; polar, positive (10%) neutral (90%)).

15 The “positively” charged amino acids are: Arginine (Arg, R; polar, positive), and Lysine (Lys, K; polar, positive).

The “negatively” charged amino acids are: Aspartic acid (Asp, D; polar, negative), and Glutamic acid (Glu, E; polar, negative).

The bispecific antibody of the present invention comprises at least one binding
20 site specifically recognizing oxMIF and one binding site specifically recognizing HSG (histamine-succinyl-glycyl) hapten, specifically having high affinity for HSG and oxMIF.

The VL and VH domains of the HSG binding site of the bispecific anti-oxMIF/anti-HSG antibody described herein are significantly modified compared to known anti-HSG variable domains. Although mouse anti-HSG mAb 679 (mo679) and a humanized mAb
25 679 (hz679) are described in US20090240037A1 and US20090246131, respectively, further modifications of the sequences were needed to provide the improved antibody as described herein. As shown below, modification of the sequences could not merely be performed according to known techniques, but also an elaborate and complex selection of the received domains was necessary to receive specific VL and VH
30 sequences which are advantageous in view of manufacturing, stability, and immunogenicity, while still showing high affinity towards moieties comprising HSG haptens.

The anti-HSG binding site of the antibody of the present invention contains a light chain variable (VL) domain comprising one of SEQ ID Nos: 17, 19, 89, 90, or 91 and a

heavy chain variable (VH) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10.

In an alternative embodiment, one or both of VL and VH comprise a sequence with at least 95%, specifically 96%, 97%, 98%, 99% sequence identity. Alternatively, one or both of VL and VH comprise 1 or 2 further amino acid substitutions. In this respect, specifically, the following applies:

Following amino acid positions of SEQ ID NO: 17 remain conserved: E105, I106, and optionally Q100.

Following amino acid positions of SEQ ID NO: 19 remain conserved: E105, I106, and optionally Q100.

Following amino acid positions of SEQ ID NO: 89 remain conserved: E105, I106, and optionally Q100.

Following amino acid positions of SEQ ID NO: 90 remain conserved: E105, I106, and optionally Q100.

Following amino acid positions of SEQ ID NO: 91 remain conserved: E105, I106, and optionally Q100. Following amino acid position of SEQ ID NO: 4 remains conserved: R19.

Following amino acid position of SEQ ID NO: 6 remains conserved: R19.

Following amino acid position of SEQ ID NO: 8 remains conserved: R19.

Following amino acid position of SEQ ID NO: 10 remains conserved: R19.

Numbering is according to Kabat.

SEQ ID NOs.: 17, 19, 89, 90, 91, 4, 6, 8, and 10 encompass all CDRs and the framework regions of the bispecific anti-tumor antigen/anti-HSG antibody described herein.

In a specific embodiment, the carboxyl terminal end of VH is linked to the amino terminal end of VL by the peptide linker Gly-Gly-Ser (G₂S), thereby forming a single-chain variable fragment (scFv). Specifically, the linker is (G₂S)_n, wherein n is 1, 2, 3, 4, or 5, specifically n is 3, 4 or 5.

In a specific embodiment, the linker is of SEQ ID NO: 39.

Specifically, said linker is used for the inventive antibody of the format scFv-Fab-Fc and IgG-central scFv.

ScFv can be directly connected to the respective constant region via a flexible linker, such as (G₄S)_n, wherein n is 1, 2, 3, 4, or 5, specifically n is 1 to 3.

In a specific embodiment, the linker is of SEQ ID NO: 42.

Specifically, said flexible linker is used for the inventive antibody of the format scFv-Fab-Fc and of the format IgG-central scFv.

The flexible linker in its turn is connected via the truncated hinge region of the SEQ ID NO: 40 to the Fc region of the antibody heavy chain (e.g., of the SEQ ID NO: 32 or SEQ ID NO: 34), specifically, as used for the inventive antibody of the format scFv-Fab-Fc and IgG-central scFv.

The VH and VL can also be connected to the respective antibody constant regions (e.g. CL kappa cross (SEQ ID NO: 38), and CH1 cross (e.g. SEQ ID NO: 30), whereas the CL kappa cross in its turn is connected via the truncated hinge (of SEQ ID NO: 40) to the Fc region of the SEQ ID NO: 32, or is connected to the truncated hinge-Fc of the SEQ ID NO: 33, specifically, as used for the inventive antibody of the format CrossMab (CH1-CL). According to a specific embodiment of the invention, the antibodies comprising the following combinations of VH and VL:

- SEQ ID NO: 4 and SEQ ID NO: 17 (VH1+VL1)
- SEQ ID NO: 4 and SEQ ID NO: 19 (VH1+VL2)
- SEQ ID NO: 4 and SEQ ID NO: 89 (VH1+VL3.1)
- SEQ ID NO: 4 and SEQ ID NO: 90 (VH1+VL4.1)
- SEQ ID NO: 4 and SEQ ID NO: 91 (VH1+VL5.1)
- SEQ ID NO: 6 and SEQ ID NO: 17 (VH2+VL1)
- SEQ ID NO: 6 and SEQ ID NO: 19 (VH2+VL2)
- SEQ ID NO: 6 and SEQ ID NO: 89 (VH2+VL3.1)
- SEQ ID NO: 6 and SEQ ID NO: 90 (VH2+VL4.1)
- SEQ ID NO: 6 and SEQ ID NO: 91 (VH2+VL5.1)
- SEQ ID NO: 8 and SEQ ID NO: 17 (VH3+VL1)
- SEQ ID NO: 8 and SEQ ID NO: 19 (VH3+VL2)
- SEQ ID NO: 8 and SEQ ID NO: 89 (VH3+VL3.1)
- SEQ ID NO: 8 and SEQ ID NO: 90 (VH3+VL4.1)
- SEQ ID NO: 8 and SEQ ID NO: 91 (VH3+VL5.1)
- SEQ ID NO: 10 and SEQ ID NO: 17 (VH4+VL1)
- SEQ ID NO: 10 and SEQ ID NO: 19 (VH4+VL2)
- SEQ ID NO: 10 and SEQ ID NO: 89 (VH4+VL3.1)
- SEQ ID NO: 10 and SEQ ID NO: 90 (VH4+VL4.1)
- SEQ ID NO: 10 and SEQ ID NO: 91 (VH4+VL5.1)

In a specific embodiment, the bispecific antibody described herein contains a variant Fc domain having a reduced or eliminated effector function and/or FcRn binding.

By "**effector function**" as used herein is meant a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. Effector
5 functions include but are not limited to ADCC, ADCP, and CDC.

By "**effector cell**" as used herein is meant a cell of the immune system that expresses one or more Fc receptors and mediates one or more effector functions. Effector cells include but are not limited to monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, B cells, large granular lymphocytes,
10 Langerhans' cells, natural killer (NK) cells, and T cells, and may be from any organism including but not limited to humans, mice, rats, rabbits, and monkeys. According to the invention, the bispecific antibodies described herein have silenced effector functions due to amino acid substitutions at selected positions in the heavy chain constant region, specifically in the Fc region. Decreased or fully silenced effector functions of these
15 antibodies due to reduced complement- and Fc γ R-mediated activities can include reduced or abolished complement dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) and/or antibody dependent cellular phagocytosis (ADCP).

The term "**Fc**" or "**Fc region**" of "**Fc domain**" (or fragment crystallizable region)
20 as used herein refers to the polypeptide comprising the constant region of an antibody excluding the first constant region immunoglobulin domain (CH1 domain) and in some cases, part of the hinge. The Fc region refers to the C- terminal region of an antibody. The Fc region is composed of two, specifically identical, protein fragments, derived from the second and third constant domains of the antibody's two heavy chains: Chain A and
25 Chain B. The second and third constant domains are known as the CH2 domain and the CH3 domain, respectively. The CH2 domain comprises a CH2 domain sequence of Chain A and a CH2 domain sequence of Chain B. The CH3 domain comprises a CH3 domain sequence of Chain A and a CH3 domain sequence of Chain B. As used herein, the Fc region includes the hinge region or a part thereof.

30 The CH2 domain of a human IgG Fc region sequence usually extends from about amino acid 231 to about amino acid 340 according to EU numbering. The CH2 domain sequence is unique in that it is not closely paired with another domain sequence. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domain sequences of an intact native IgG molecule.

The CH3 domain comprises the stretch of residues C-terminal to a CH2 domain sequence in an Fc region sequence (i.e. from about amino acid residue 341 to about amino acid residue 447 of an IgG according to EU numbering).

5 A "**functional Fc region**" or "functional Fc domain" possesses the "effector functions" and the FcRn binding of a native Fc region. Exemplary "effector functions" include C1 q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays known in the art and as herein
10 disclosed.

A "**native Fc region**" or "native Fc domain" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region, native sequence human IgG3 Fc region and native sequence human IgG4 Fc region as well as naturally occurring variants
15 thereof.

A "**variant Fc region**" or "variant Fc-domain" comprises an amino acid sequence which differs from that of a native Fc region sequence by virtue of "one or more amino acid substitutions". The variant Fc region sequence has at least one amino acid
20 substitution compared to a native Fc region sequence or to the Fc region sequence of a parent polypeptide, e.g. from about one to about twenty amino acid substitutions, and preferably from about one to about seventeen amino acid substitutions in a native Fc region sequence or in the Fc region sequence of the parent polypeptide. In certain embodiments, the variant Fc region sequence herein possesses at least about 80%
25 identity with a native Fc region sequence and/or with an Fc region sequence of a parent polypeptide, and most preferably at least about 90% identity therewith, more preferably at least about 95% identity therewith.

In a specific embodiment, the amino acid substitutions are at any one or more of positions E233, L234, L235, G236, I253, G237, P238, D265, S267, H268, N297, S298,
30 T299, H310, E318, L328, P329, A330, P331 and H435 with reference to IgG1, or SEQ ID NO: 31 and according to EU numbering.

In a further specific embodiment, the amino acid substitutions are at any one or all of positions L234, L235, H310, in the CH2 domain, and H435 in the CH3 domain, all with reference to IgG1 (SEQ ID NO: 31) according to EU numbering. More specifically,

the substitutions are L234A, L235A, H310A and/or H435Q. These residues are located in the Fc region and can lead to a near complete inhibition of FcγR interaction and thus can result in almost complete or complete Fc silencing.

Modifications of the Fc region resulting in decreased or silenced Fc regarding effector functions are known in the art and are described in Saunders K., 2019 and Liu R. et al., 2020.

In an alternative embodiment, the amino acid substitutions are at any one or all of positions L234F, H268Q, K274Q, Y296F, A327G, A330S, P331S in the CH2 domain and R355Q, K409R, Q419E, P445L in the CH3 domain.

Specifically, the Fc silenced bispecific antibody described herein comprises one or more of the following combinations of amino acid substitutions or deletions:

- i) L235, G237 and E318, specifically L235A, G237A and E318A;
- ii) L234, L235, specifically L234A, L235A;
- iii) S228, L235, specifically S228P, L235E;
- iv) G236, L328, specifically G236R, L328R;
- v) S298, T299, specifically S298G, T299A;
- vi) L234, L235, P331, specifically L234F, L235E, P331S;
- vii) H268, V309, A330, P331, specifically H268Q, V309L, A330S, P331S;
- viii) E233, L234, L235, G236, S267, specifically E233P, L234V, L235A, G236del, S267K;
- ix) L234, L235, P329, specifically L234A, L235A, P329G;
- x) V234, G237, P238, H268, A330, P331, specifically V234A, G237A, P238S, H268A, A330S, P331S;
- xi) L234, L235, D265, specifically L234F, L235E, D265A;
- xii) D265, specifically D265A;
- xiii) G237, specifically G237A;
- xiv) E318, specifically E318A;
- xv) E233, specifically E233P,
- xvi) G236, L328, specifically G236R, L328R;
- xvii) L235, specifically L235E;
- xviii) L234, L235, P331, specifically L234Q, L235F, P331S;
- xix) L234, L235, G237, P238, H268, A330, P331, specifically L234A, L235A, G237A, P238S, H268A, A330S, P331S;

xx) N297, specifically N297A, N297Q or N297G, leading to an aglycosylated antibody.

Glycosylation, O- and N-glycosylation, is a post-translational modification of Abs, which can be regulated by a range of B cell stimuli, including environmental factors, such as stress or disease, cytokine activity, and innate immune signaling receptors, such as Toll-like receptors. Glycosylation pattern of the parent antibody can be modified by methods well known in the art. Specifically, O-linked glycosylation sites are located in the CH2 and hinge regions.

Specifically, the bispecific antibody described herein comprises one or more of the following combinations of amino acid substitutions:

- i) I253A;
- ii) H310A;
- iii) H435, specifically H435A, H435Q or H435R;
- iv) I253A and H310A;
- v) I253A and H310A, and one of H435Q, H435A, and H435R;
- vi) H310A and one of H435Q, H435A, and H435R.

The herein described silencing and further mutations in the CH domains, however, can also be introduced into the Fc of wild type IgG2, IgG3 or IgG4 at corresponding positions according to EU numbering.

The term “**aglycosylated**” indicates that the Fc region is not glycosylated. All human constant regions of the IgG isotype are known to be glycosylated at the Asp residue at position 297, which makes up part of the N-glycosylation motif Asp 297-X 298-Ser 299 or Thr 299, where X is the residue of any amino acid except proline. The glycan has a heptasaccharide core and variable extensions, such as fucose, galactose and/or sialic acid. The antibody of the invention may thus be aglycosylated by the replacement of Asp 297 in such a constant region with another amino acid which cannot be glycosylated or deglycosylated by enzymatic means. Any other amino acid residue can potentially be used, but Ala is the most preferred. Alternatively, glycosylation at Asp 297 can be prevented by altering one of the other residues of the motif, e.g. by replacing residue 298 by Pro, or residue 299 by any amino acid other than Ser or Thr. Techniques for performing this site directed mutagenesis are well known to those skilled in the art and may for example be performed using a commercially available site directed mutagenesis kit.

The term “**silenced Fc**” refers to an Fc region of an antibody whose effector function and/or FcRn binding is reduced or eliminated due to amino acid substitutions or modification of the glycosylation pattern resulting in modified glycan that reduce or eliminate binding of the antibody to any of FcγR, such as FcγRIIaH, FcγRIIaR, FcγRIIb
5 FcγRIIIaF, FcγRIIIaV, and FcγRIa and/or FcRn receptors and to complement factor C1 q protein. Such reduction or elimination of this binding results in reduction or elimination of effector functions and/or FcRn binding typically mediated by the wild-type IgG Fc region.

If FcγR binding, such as one any FcγRIIa, FcγRII FcγRIIIa, and FcγRIa and/or
10 FcRn receptors and to complement factor C1 q protein is completely abolished, the term “Fc null” may be used herein.

A great extent of Fc silencing can be achieved by combining mutations L234 and L235, these residues being located close to the hinge area and reducing FcγR binding when substituted by alanine. As an example, the combination of L234A and L235A with
15 P329G can lead to a near complete inhibition of FcγR interaction for all FcγR isoforms.

A Fc silenced bispecific antibody described herein with greatly reduced, silenced, negligible or ablated FcγR binding affinity and C1q binding affinity is one which has diminished FcγR binding activity and C1q binding activity compared to a parent polypeptide or to a polypeptide comprising a native Fc region sequence. In some
20 embodiments, an Fc silenced bispecific antibody with greatly reduced, silenced, negligible or ablated FcR binding affinity and C1q binding affinity has also greatly reduced, silenced, negligible or ablated ADCC, ADCP and CDC activity compared to a parent polypeptide or to a polypeptide comprising a native Fc region sequence. A Fc silenced bispecific antibody as described herein which displays decreased or
25 undetectable binding to FcγR may bind all FcγRs with lower affinity than the parent polypeptide. An exemplary parent polypeptide can be the antibody Imalumab. Such variants which display decreased binding to an FcγR may possess little or no appreciable binding to an FcγR. In one specific embodiment, the variant displays 0-20% binding to the FcγR compared to a native IgG Fc region, e.g. as measured by change in
30 equilibrium constant. In one embodiment, the variant displays 0-10% binding to the FcγR compared to a native IgG Fc region. In one embodiment, the variant displays 0-5% binding to the FcγR compared to a native IgG Fc region. In one embodiment, the variant displays 0-1 % binding to the FcγR compared to a native IgG Fc region.

Antibodies described herein with silenced complement activities can be determined by cell-based CDC assays and reduced or abolished binding to C1q determined by i.e. SPR or ELISA.

Decreased or silenced CDC activity is determined to be at least 1.5-fold, specifically at least 2-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, more specifically at least 10-fold downregulated compared to a reference, i.e. unmodified, wild type Fc.

Decreased ADCC or ADCP activity is determined to be at least 2-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, more specifically at least 10-fold decreased potent compared to a reference antibody, i.e. unmodified, wild type Fc.

In a specific embodiment, anti-tumor antigen/anti-HSG antibody show reduced binding to FcRn due to amino acid substitutions at selected positions in the heavy chain constant region. Decreased binding of the Fc silenced bispecific antibody of the present invention to the FcRn leads to reduced half-life in the circulation and faster in vivo clearance. FcRn binding and in vivo clearance/half-life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al., 2006).

The Fc domain of the bispecific antibody described herein, exhibiting decreased FcRn binding compared to an anti-tumor antigen/anti-HSG antibody comprising the wild-type IgG Fc region may preferably comprise an Fc domain comprising one, two or three amino acid substitutions at any one of positions I253, H310, and H435.

The bispecific antibodies described herein may also carry the L234A/L235A („LALA“) mutations at the beginning of the CH2 region of the heavy chains. Introduction of L234A/L235A mutations was demonstrated to result in nearly complete elimination of mAb binding to FcγRs and strongly reduced binding to the complement (Lo M. et al., 2017, Wang X. et al., 2018, Zhou Q. et al., 2020).

Fc silencing also allows to minimize potential unwanted effects during radio-imaging, specifically when performing pre-targeting, through the binding of the antibodies described herein to Fcγ receptor- bearing immune cells and complement.

Decreased FcRn binding (i.e. affinity) is determined to be at least 2-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, more specifically at least 10-fold decreased binding (i.e. affinity) compared to a reference antibody, i.e. unmodified, wild type antibody.

According to a specific embodiment, two different heavy chains may be combined in the antibody described herein. Heterodimerization of two different heavy chains can be achieved by using techniques such as engineering of heterodimeric Fc variants through the replacement of homodimer-favoring interactions at the CH3 domain

interface with heterodimer-favoring interactions due to introduction of asymmetric mutations in each CH3 domain which promotes assembly of HCs from different antibodies. Therefore, structure-based rational design and directed evolution can be used, such that the variant pair thermodynamically favors the formation of heterodimers over the homodimers. Strategies known from the art are, e.g., symmetric-to-asymmetric steric complementarity design (e.g., KiH, HA-TF, and ZW1); charge-to-charge swap (e.g., DD-KK); charge-to-steric complementarity swap plus additional long-range electrostatic interactions (e.g., EW-RVT); and isotype strand swap (e.g., strand-exchange engineered domain (SEED)).

Knob-into-hole (KiH) technology: One heavy chain is carrying in its CH3 region the „knob“ mutation T366W and S354C mutation for disulfide bond stabilization (Merchant A.M. et al., 1998), specifically this CH3 region carries the anti-tumor-antigen binding site, e.g. the anti-oxMIF binding site. The heavy chain carrying in its CH3 region the „hole“ mutations T366S/L368A/Y407V and Y349C mutation for disulfide bond stabilization (Merchant A.M. et al., 1998), specifically this CH3 region carries the anti-HSG binding site.

According to a specific embodiment of the invention, the antibody described herein comprises SEQ ID NO: 35 and 32.

According to an embodiment of the invention, the antibody described herein comprises SEQ ID NO: 36 and 33 or SEQ ID NO: 36 and 34.

The antibody described herein can be used for detecting and localizing a tumor having on its cell surface a tumor-associated or tumor-specific antigen.

The anti-tumor antigen binding site can specifically target and bind to any tumor antigen or tumor associated antigen and may include, but is not limited to melanoma cell surface antigens, breast cancer cell surface antigens such as CA15-3, lung cancer cell surface antigens, colorectal cancer cell surface antigens, gastric cancer cell surface antigens, pancreatic cancer cell surface antigens, glioma cell surface antigens, common sarcoma cell surface antigens, gastrointestinal cancer cell surface antigens, brain tumor cell surface antigens, esophageal cancer cell surface antigens, common epithelial cancer cell surface antigens, osteosarcoma cell surface antigens, fibrosarcoma cell surface antigens, urinary bladder cancer cell surface antigens, prostatic cancer cell surface antigens, renal cancer cell surface antigens, ovarian cancer cell surface antigens, testicular cancer cell surface antigens, endometrial cancer cell surface antigens, cervical cancer cell surface antigens, Hodgkin's disease cell surface antigens,

lymphoma cell surface antigens, leukemic cell surface antigens, trophoblastic tumor cell surface antigens, tumor necrosis antigens, oxMIF. Where the disease state is cancer, for example, many antigens expressed by or otherwise associated with tumor cells are known in the art, including but not limited to, carbonic anhydrase IX, alpha-fetoprotein, 5 α -actinin-4, A3, antigen specific for A33 antibody, ART-4, B7, Ba 733, BAGE, BrE3-antigen, CEA, Cancer antigen CA-125, PSA, CAP-1, CASP-8/m, CCL19, CCL21, CEA CEACAM5, CEACAM6, CEACAM8, c-met, CDK-4/m, CDKN2A, CXCR4, CXCR7, CXCL12, HIF-1 α , colon-specific antigen-p (CSAp), Flt-1, Flt-3, folate receptor, G250 antigen, GAGE, gp100, GRO- β , HLA-DR, HM1.24, human chorionic gonadotropin 10 (HCG) and its subunits, HER2/neu, HMGB-1, hypoxia inducible factor (HIF-1), HSP70-2M, HST-2, Ia, IGF-1R, IFN- γ , IFN- α , IFN- β , IL-2, IL-4R, IL-6R, IL-13R, IL-15R, IL-17R, IL-18R, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, IL-25, insulin-like growth factor-1 (IGF-1), KC4-antigen, KS-1-antigen, KS1-4, Le-Y, LDR/FUT, HER2, DAM, EGFR, EGFRvIII, EGP-1, EGP-2, ELF2-M, EpCAM, Mesothelin (MSLN), oxidized macrophage migration 15 inhibitory factor (oxMIF), MAGE, MAGE-3, MART-1, MART-2, NY-ESO-1, TRAG-3, mCRP, MCP-1, MIP-1A, MIP-1B, Folate Receptor alpha (FR α), EGFR, Trop-2, MUC1, MUC2, MUC3, MUC4, MUC5, MUM-1/2, MUM-3, NCA66, NCA95, NCA90, pancreatic cancer mucin, placental growth factor, p53, PLAGL2, prostatic acid phosphatase, PSA, PRAME, PSMA, PIGF, ILGF, ILGF-1R, RS5, RANTES, T101, SAGE, S100, survivin, 20 survivin-2B, TAC, TAG-72, tenascin, Thomson-Friedenreich antigens, VEGFR, ED-B fibronectin, WT-1, 17-1A-antigen, complement factors C3, C3a, C3b, C5a, C5, an angiogenesis marker, bcl-2, bcl-6, Kras, cMET, an oncogene marker and an oncogene product, tumor necrosis antigens, TNF- α , TRAIL receptor (R1 and R2), NCA-90, NCA-95, transmembrane activator and CAML-interactor (TACI), B-cell maturation antigen 25 (BCMA), APRIL, TALL-I (also called BLyS or BAFF), , CD1, CD1a, CD2, CD3, CD4, CD5, CD8, CD11A, CD14, CD15, CD16, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD29, CD30, CD32b, CD33, CD37, CD38, CD40, CD40L, CD45, CD46, CD52, CD54, CD55, CD59, CD64, CD66a-e, CD67, CD70, CD74, CD79a, CD80, CD83, CD95, CD126, CD133, CD138, CD147, CD154, CXCR4, CD3, ADAM17, CD2, CD6, CD11a, 30 CD11b, CD16, CD16b, CD28, CD30, CD32a, CD44, CD56, CD57, CD64, CD69, CD74, CD89, CD90, CD137, CD177, CDC27, HLA-DR alpha-chain, KIR, LSEctin or SLC44A2, B7, Ia, II, HM1.24, HLA-DR, tenascin, VEGF, PIGF, ED-B fibronectin, an oncogene, an oncogene product (e.g., c-met or PLAGL2), IL-2, T101, TAG, and the like.

According to a specific embodiment, the anti-tumor antigen binding site of the antibody described herein is specifically recognizing oxMIF.

oxMIF antibodies have been described in PCT/EP2021/077106 and PCT/EP2022/052463.

5 The oxMIF binding site is specific for the oxidized form of MIF, i.e. specifically for human oxMIF, and does not show substantial cross-reactivity to reduced MIF. oxMIF is the disease-related structural isoform of MIF which can be specifically and predominantly detected in the circulation of subjects with inflammatory diseases and in tumor tissue of cancer patients.

10 oxMIF binding specificity can be determined by any assay appropriate for determining selective binding to oxMIF, such as any competition assay against a control antibody, such as Imalumab, with respect to binding to oxMIF or various assays known in the art and as herein disclosed.

15 The bispecific antibody of the invention comprises at least one binding site specifically recognizing oxMIF and, according to a specific embodiment, exhibits reduced aggregation propensity and reduced hydrophobicity due to targeted amino acid substitutions in the variable heavy and light domains in comparison to the unmodified antibody lacking said amino acid substitutions.

20 Reduction of aggregation potential is due to amino acid substitutions at selected positions within the variable domains of the antibody described herein.

25 The level of antibody aggregation can be measured using a variety of known techniques including mass spectrometry, size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC), dynamic light scattering (DLS), light obscuration (LO), dynamic imaging particle analysis (DIPA) techniques such as microflow imaging (MFI), and Coulter counter (CC), differential scanning fluorometry (DSF).

30 Reduced hydrophobicity and reduced aggregation potential as used herein refers to a reduction of the surface hydrophobicity and a reduced aggregation potential of the antibody compared to a reference antibody such as antibody Imalumab, published in the Proposed INN List 111 (WHO Drug Information, Vol. 28, No. 2, 2014), but lacking the C-terminal lysine. Measurement can be performed using a variety of known techniques including but not limited to hydrophobic interaction chromatography (HIC) or affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS, Estep P. et al., 2015).

In an embodiment, the oxMIF binding site of antibody of the invention specifically comprises a light chain variable domain having SEQ ID NO: 45 with one or more, specifically 1, 2, 3, 4, or 5 amino acid substitutions at positions M30, F49, A51, P80, W93, specifically M30L, F49Y, A51G, P80S, W93F, more specifically F49Y, A51G, W93F according to the Kabat numbering, or a light chain variable domain with at least 95%, specifically at least 96%, 97%, 98% or 99% sequence identity to SEQ ID NO: 45 comprising tyrosine at position 36, and furthermore at least one, specifically 1, 2, 3, 4, 5, of the amino acid substitutions at positions M30, F49, A51, P80, W93, specifically M30L, F49Y, A51G, P80S, and W93F, more specifically F49Y, A51G, and W93F, either in combination with a heavy chain variable domain comprising SEQ ID NO: 46 or in combination with a heavy chain variable domain comprising SEQ ID NO: 46 with the amino acid substitutions at positions L5, and/or W97, specifically L5Q and/or W97Y, according to the numbering of Kabat, or a heavy chain variable domain having 96%, 97%, 98% or 99% sequence identity to SEQ ID NO: 46 comprising the amino acid substitutions at positions L5 and/or W97, specifically L5Q and/or W97Y.

According to a specific embodiment, amino acid W93 is replaced by F, Y, or H.

In a further embodiment, the anti-oxMIF antibody of the invention having reduced aggregation potential and reduced hydrophobicity specifically comprises a heavy chain variable domain comprising SEQ ID NO: 46 and an amino acid substitution at position W97, specifically W97Y, or an amino acid substitution at L5, specifically L5Q, or amino acid substitutions L5Q and W97Y, or a heavy chain variable domain comprising 96%, 97%, 98% or 99% sequence identity with SEQ ID NO: 46 and further comprising amino acid substitution W97Y, optionally in combination with L5Q.

According to a specific embodiment, amino acid W97 is replaced by F, Y, or H.

In a preferred embodiment, the anti-oxMIF antibody of the invention having reduced aggregation potential and reduced hydrophobicity specifically comprises amino acid substitutions at positions W93 and W97.

The tyrosine at position 36 of the light chain is specifically kept unmodified to preserve binding properties of the antibody described herein. Any modifications at said amino acid position may result in unwanted impaired binding properties.

In a specific embodiment, the antibody described herein comprises,

- a light chain variable domain comprising SEQ ID NO: 27, or a light chain variable domain comprising SEQ ID NO: 27 and further comprising amino acid substitutions M30L and/or P80S, and

- a heavy chain variable domain comprising SEQ ID NO: 46, specifically with amino acid substitutions L5Q and/or W97Y,

wherein the amino acid positions are numbered according to Kabat.

The term “**antibody**” herein is used in the broadest sense and encompasses polypeptides or proteins that consist of or comprise antibody domains, which are understood as constant and/or variable domains of the heavy and/or light chains of immunoglobulins, with or without a linker sequence. The term also encompasses fusion proteins, such as fusions with immunotoxins or antibody conjugates, such as antibody drug conjugates binding to HSG and oxMIF.

Antibody domains may be of native structure or modified by mutagenesis or derivatization, e.g. to modify the antigen binding properties or any other property, such as stability or functional properties, such as binding to the Fc receptors, such as FcRn and/or Fc-gamma receptor. Polypeptide sequences are considered antibody domains, if they comprise a beta-barrel structure consisting of at least two beta-strands of an antibody domain structure connected by a loop sequence.

It is understood that the term “antibody” includes antigen binding derivatives, variants, and fragments thereof. A derivative or variant is any combination of one or more antibody domains or antibodies of the invention and/ or a fusion protein in which any domain of the antibody of the invention may be fused at any position of one or more other proteins, such as other antibodies or antibody formats, e.g. a binding structure comprising CDR loops, a receptor polypeptide, but also ligands, scaffold proteins, enzymes, labels, toxins and the like.

The term “antibody” shall particularly refer to polypeptides or proteins that exhibit binding properties to the target tumor antigen, specifically to oxMIF, and HSG.

The terms “**antibody fragment, antigen-binding fragment, antigen binding variant or antibody variant**” can be used interchangeably and refers to a molecule other than an intact antibody that comprises an antigen binding portion of an intact antibody that binds the antigen to which the intact antibody binds and multispecific antibodies formed from antibody fragments or variants and further comprises a variant Fc region as described herein. Examples of antigen portions include but are not limited to Fv, Fab, Fab', Fab'-SH, single chain antibody molecules (e.g. scFvs) diabodies, cross-Fab fragments; linear antibodies. According to the invention, the antibody fragment or variant is fused to a silenced Fc-portion or silenced Fc-domains by a hinge region and/or a linker, specifically a hinge region preceded by a linker, (e.g. (scFv) -Fc, (scFv)₂-Fc,

scFv/scFv-Fc, Fab/scFv-Fc, Fab/(scFv)₂-Fc, Fab/Fab-scFv-Fc (IgG-central scFv), Fab/Fab-crossFab-Fc, IgG-scFv and IgG-(scFv)₂,

In addition, antibody fragments comprise single chain polypeptides having the characteristics of a VH domain, namely being able to assemble with a VL domain, or of
5 a VL domain, namely being able to assemble with a VH domain to a functional antigen binding site and thereby providing the antigen binding property of full-length antibodies. Antibody fragments as referred herein also encompass silenced Fc domains comprising one or more structural loop regions containing antigen binding regions such as FcabTM or full-length antibody formats with IgG structures in which the silenced Fc region has
10 been replaced by an FcabTM containing second distinct antigen binding site.

As used herein, "**Fab fragment or Fab**" refers to an antibody fragment comprising a light chain fragment comprising a VL domain and a constant domain of a light chain (CL), and a VH domain and a first constant domain (CH1) of a heavy chain. The antibodies of the invention can comprise at least one Fab fragment, wherein either the
15 variable regions or the constant regions of the heavy and light chain are exchanged. Due to the exchange of either the variable regions or the constant regions, said Fab fragment is also referred to as "cross-Fab fragment" or "crossover Fab fragment". Two different chain compositions of a crossover Fab molecule are possible and comprised in the antibodies of the invention: The variable regions of the Fab heavy and light chain
20 can be exchanged, i.e. the crossover Fab molecule comprises a peptide chain. According to the invention, the Fab is fused to a silenced Fc-portion or silenced Fc-domains by a hinge region.

As used herein, "**Fab arm**" refers to a Fab fragment fused to a silenced Fc-portion or silenced Fc-domains by a hinge region.

25 The term "**functional variant**" or "functionally active variant" also includes naturally occurring allelic variants, as well as mutants or any other non-naturally occurring variants. As is known in the art, an allelic variant, or also referred to as homologue, is an alternate form of a nucleic acid or peptide that is characterized as having a substitution, deletion, or addition of one or more nucleotides or amino acids
30 that does essentially not alter the biological function of the nucleic acid or polypeptide. Specifically, a functional variant may comprise a substitution, deletion and/or addition of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid residues, or a combination thereof, which substitutions, deletions and/or additions are conservative modifications and do not alter the antigen binding properties. Specifically,

a functional variant as described herein comprises no more than or up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid substitutions, deletions and/or additions, which are conservative modifications and do not alter the antibody's function. Specifically, a functionally active variant as described herein comprises up to 5 15, preferably up to 10 or 5, amino acid substitutions, deletions and/or additions, which are conservative modifications and do not alter the antibody's function.

Functional variants may be obtained by sequence alterations in the polypeptide or the nucleotide sequence, e.g. by one or more point mutations, wherein the sequence alterations retain or improve a function of the unaltered polypeptide or the nucleotide 10 sequence, when used in combination of the invention. Such sequence alterations can include, but are not limited to, (conservative) substitutions, additions, deletions, mutations and insertions. Conservative substitutions are those that take place within a family of amino acids that are related in their side chains and chemical properties. Examples of such families are amino acids with basic side chains, with acidic side 15 chains, with non-polar aliphatic side chains, with non-polar aromatic side chains, with uncharged polar side chains, with small side chains, with large side chains etc.

According to a specific embodiment, the antibodies described herein may comprise one or more tags for purification and/or detection, such as but not limited to affinity tags, solubility enhancement tags and monitoring tags.

20 Specifically, the affinity tag is selected from the group consisting of poly-histidine tag, poly-arginine tag, peptide substrate for antibodies, chitin binding domain, RNase S peptide, protein A, β -galactosidase, FLAG tag, Strep II tag, streptavidin-binding peptide (SBP) tag, calmodulin-binding peptide (CBP), glutathione S-transferase (GST), maltose-binding protein (MBP), S-tag, HA tag, and c-Myc tag, specifically the tag is a His tag 25 comprising one or more H, such as a hexahistidine tag.

By "**fused**" or "**connected**" or "**conjugated**" is meant that the components (e.g., a Fab molecule and an Fc domain subunit) are linked by peptide bonds, either directly or via one or more peptide linkers.

The term "**linker**" as used herein with respect to the linkage between the HSG 30 hapten and a chelator and/or a therapeutic/diagnostic agent refers to a peptide linker and is preferably a peptide with an amino acid sequence with a length of 2, 3, 4, 5, 6, 7 or more amino acids, preferably with a length of 3-15, more preferably of 3-5 amino acids. Specific linkers are described herein, such as SEQ ID NOs: 39, and 42 and can be of variable length.

The term "**immunoglobulin**" refers to a protein having the structure of a naturally occurring antibody. For example, immunoglobulins of the IgG class are heterotetrameric glycoproteins of about 150,000 daltons, composed of two light chains and two heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3), also called a heavy chain constant region. Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain, also called a light chain constant region. An immunoglobulin of the IgG class essentially consists of two Fab molecules and an Fc domain, linked via the immunoglobulin hinge region. The heavy chain of an immunoglobulin may be assigned to one of five types, called α (IgA), δ (IgD), ϵ (IgE), γ (IgG), or μ (IgM), some of which may be further divided into subtypes, e.g. γ_1 (IgG₁), γ_2 (IgG₂), γ_3 (IgG₃), γ_4 (IgG₄), α_1 (IgA₁) and α_2 (IgA₂). The light chain of an immunoglobulin may be assigned to one of two types, called kappa (κ) and lambda (λ).

The term "**chimeric antibody**" refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species, usually prepared by recombinant DNA techniques. Chimeric antibodies may comprise a rabbit or murine variable region and a human constant region. Chimeric antibodies are the product of expressed immunoglobulin genes comprising DNA segments encoding immunoglobulin variable regions and DNA segments encoding immunoglobulin constant regions. Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques are well known in the art (Morrison, S.L., et al., 1984).

A "**human antibody**" possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. As also mentioned for chimeric and humanized antibodies, the term "human antibody" as used herein also comprises such antibodies which are modified in the constant region e.g. by "class switching" i.e. change or mutation of Fc parts (e.g. from IgG₁ to IgG₄ and/or IgG₁/IgG₄ mutation.)

The term "**recombinant human antibody**", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell such as a HEK cell, NS0 or CHO cell or from an animal (e.g. a mouse) that is transgenic for human immunoglobulin genes or antibodies expressed using a recombinant expression vector transfected into a host cell. The amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germ line sequences, may not naturally exist within the human antibody repertoire in vivo.

A "**human consensus framework**" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as described in Kabat et al., 1991.

A "**humanized**" antibody refers to a chimeric antibody comprising amino acid residues from non-human hypervariable regions (HVRs) and amino acid residues from human framework regions (FRs) which has undergone humanization. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. Specifically, forms of humanized antibodies are encompassed by the present invention in which the constant region has been additionally modified or changed from that of the original antibody to generate the new properties, i.e. in regard to reduced or abolished C1q binding and/or FcR binding.

Methods and details of humanization of the anti-HSG are described herein, specifically in the examples.

The term "**bispecific**" as used herein shall refer to the binding reaction at least with an anti-tumor antigen, e.g. an oxMIF antigen, and a further antigen, a HSG hapten antigen. A bispecific antibody specifically can comprise at least two sites with specific binding properties, wherein two different target antigens, at least one tumor antigen and HSG are recognized by the antibody. The bispecific antibody format comprises two binding sites, wherein each of the binding sites is capable of specifically binding a tumor antigen and HSG. Alternatively, a further exemplary bispecific format may comprise

more than two binding sites, e.g. 3, 4, 5 or more binding sites, wherein one or more binding sites bind to one or more same or different tumor antigens and one or more binding sites are capable of specifically binding to HSG.

"**Bispecific antibodies**" according to the invention are antibodies which have two
5 different binding specificities. Bispecific antibodies can be prepared as full-length antibodies as described herein, or antibody fragments still comprising a silenced Fc region. Immunoglobulin Fc heterodimers may be engineered through modifications to the CH3 domain interface, with different mutations on each domain such that the engineered Fc fragments, carrying the CH3 variant pair, preferentially form heterodimers
10 rather than homodimers (Ha J-H. et al., 2016). Examples of bispecific antibody formats can be, but are not limited to bispecific IgGs (BsIgG), IgGs appended with an additional antigen-binding moiety, BsAb fragments, bispecific fusion proteins, BsAb conjugates, hybrid bsIgGs, modified Fc fusion proteins, appended IgGs-HC fusions, appended IgGs-LC fusions, appended IgGs-HC& LC fusions, Fc fusions, CH3 fusions, F(ab')₂ fusions,
15 CH1/CL, modified IgGs, Fc-modified IgGs, diabodies, etc. as described in Spiess C. et al., 2015, and Brinkmann U. and Kontermann R.E., 2017.

In an alternative embodiment encompassing bispecific antibodies described herein, the term "**IgG-scFv**" refers to a kind of bispecific antibodies which is engineered for bispecificity by fusing one scFv to a monospecific Immunoglobulin G (IgG). According
20 to an embodiment of the invention, the bispecific antibodies are Fc silenced, i.e. they comprise a variant Fc region of a wild-type human IgG with one or more amino acid substitutions or a glycosylation modification described herein. The specificity of the IgG can be for a tumor antigen and the specificity of the scFv can be for HSG hapten or vice versa. Furthermore, either the amino terminus or the C terminus of one of the light or
25 heavy chains can be appended with an scFv, which leads to the production of diverse types of IgG-scFv bispecific antibodies (BsAbs): (i) IgG(H)-scFv, an scFv linked to the C terminus of one of the full-length IgG HC; (ii) scFv-(H)IgG, which is same like IgG(H)-scFv, except that the scFv is linked to the HC N-terminus; (iii) IgG(L)-scFv or (iv) scFv-(L)IgG, the scFv connected to the C or N-terminus of the IgG light chain, which forms
30 the IgG(L)-scFv or scFv-(L)IgG, respectively. Specifically, the IgG-scFv is in the range of 165 kDa to 185 kDa, specifically it is about 175 kDa.

In a specific embodiment, the term Fab/bs(scFv)₂-Fc refers to bispecific antibody which is an IgG having one Fab arm replaced by a bs(scFv)₂, while the second IgG arm is preserved.

In a specific embodiment, the term Fab/scFv-Fc refers to a bispecific antibody which is an IgG having one Fab arm replaced by a scFv, while the second IgG arm is preserved. Antibody C0181, as described herein and as schematically shown in Figure 1 serves as a non-limiting example of a Fab/scFv-Fc.

5 In a specific embodiment, the term Fab/Fab-scFv-Fc (IgG-central scFv) refers to bispecific antibody which is an IgG having one Fab arm replaced by a Fab-scFv, while the second IgG arm is preserved.

The term "**CrossMab**" (where Mab refers to monoclonal antibody) is a format of bispecific Abs derived from independent parental antibodies. Heavy chain mispairing is avoided by applying the knobs-into-holes (KIH) method. Light chain mispairing is avoided as the bispecific antibody is produced with antibody domain exchange whereas either the variable domains or the constant domains (CL and CH1) of one Fab arm are swapped between the light and heavy chains. This "crossover" keeps the antigen-binding affinity and also preserves the two different arms in order to avoid light-chain mispairing. Examples of CrossMabs can be, but are not limited to Fab, VH-VL and CH1-CL exchanged in different regions. In CrossMabs Fabs the full VH-CH1 and VL-CL regions are exchanged; in CrossMab VH-VL format only the VH and VL regions are exchanged; in CrossMab CH1-CL1 format the CH1 and CL regions of bispecific antibody are exchanged. Specifically, the CrossMab is of about 150kDa.

20 The term "**antigen**" as used herein interchangeably with the term "**target**" or "**target antigen**" shall refer to a whole target molecule or a fragment of such molecule recognized by an antibody binding site. Specifically, substructures of an antigen, e.g. a polypeptide or carbohydrate structure, generally referred to as "epitopes", e.g. B-cell epitopes or T-cell epitope, which are immunologically relevant, may be recognized by such binding site.

25 The term "**epitope**" as used herein shall, in particular, refer to a molecular structure which may completely make up a specific binding partner or be part of a specific binding partner to a binding site of an antibody format of the present invention. An epitope may either be composed of a carbohydrate, a peptidic structure, a fatty acid, an organic, biochemical, or inorganic substance, or derivatives thereof, and any combinations thereof. If an epitope is comprised in a peptidic structure, such as a peptide, a polypeptide, or a protein, it will usually include at least 3 amino acids, specifically 5 to 40 amino acids, and specifically less than 10 amino acids, specifically between 4 to 10 amino acids. Epitopes can be either linear or conformational epitopes.

A linear epitope is comprised of a single segment of a primary sequence of a polypeptide or carbohydrate chain. Linear epitopes can be contiguous or overlapping. Conformational epitopes are comprised of amino acids or carbohydrates brought together by folding the polypeptide to form a tertiary structure and the amino acids are not necessarily adjacent to one another in the linear sequence. An exemplary oxMIF epitope may be sequence EPCALCS (SEQ ID NO: 53) located within the central region of oxMIF.

The term “**antigen binding domain**” or “binding domain” or “binding-site” refers to the part of an antigen binding moiety that comprises the area which specifically binds to and is complementary to part, or all of an antigen. Where an antigen is large, an antigen binding molecule may only bind to a particular part of the antigen, which part is termed an epitope. An antigen binding domain may be provided by, for example, one or more antibody variable domains (also called antibody variable regions). Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

The term “**binding site**” as used herein with respect to the antibody of the present invention refers to a molecular structure capable of binding interaction with an antigen. Typically, the binding site is located within the complementary determining region (CDR) of an antibody, herein also called “a CDR binding site”, which is a specific region with varying structures conferring binding function to various antigens. The varying structures can be derived from natural repertoires of antibodies, e.g. murine or human repertoires, or may be recombinantly or synthetically produced, e.g. by mutagenesis and specifically by randomization techniques. These include mutagenized CDR regions, loop regions of variable antibody domains, in particular CDR loops of antibodies, such as CDR1, CDR2 and CDR3 loops of any of VL and/or VH antibody domains. The antibody format as used according to the invention typically comprises one or more CDR binding sites, each specific to an antigen.

The oxMIF binding site of the antibody described herein is specific for the oxidized form of MIF, i.e. for animal, specifically for mammalian oxMIF, such as but not limited to mouse, rat, monkey, and human, specifically for human oxMIF, but does not show substantial cross-reactivity to reduced MIF. In one embodiment, the humanized or human anti-oxMIF binding site comprises one or more (e.g., all three) light chain complementary determining regions of a humanized or human anti-oxMIF binding domain described herein.

The term “**specific**” as used herein shall refer to a binding reaction which is determinative of the cognate ligand of interest in a heterogeneous population of molecules. Herein, the binding reaction is at least with an oxMIF antigen. Thus, under designated conditions, e.g. immunoassay conditions, the antibody that specifically binds to its particular target does not bind in a significant amount to other molecules present in a sample, specifically it does not show substantial cross-reactivity to reduced MIF.

A specific binding site is typically not cross-reactive with other targets. Still, the binding site may specifically bind to one or more epitopes, isoforms, or variants of the target, or be cross-reactive to other related target antigens, e.g., homologs or analogs.

The specific binding means that binding is selective in terms of target identity, high, medium, or low binding affinity or avidity, as selected. Selective binding is usually achieved if the binding constant or binding dynamics to a target tumor antigen such as e.g. oxMIF, or to HSG hapten is at least 10-fold different, preferably the difference is at least 100-fold, and more preferred a least 1000-fold compared to binding constant or binding dynamics to an antigen which is not the target antigen.

The term “**valent**” as used within the current application denotes the presence of a specified number of binding sites in an antibody molecule. As such, the terms “bivalent”, “tetravalent”, and “hexavalent” denote the presence of two binding sites, four binding sites, and six binding sites, respectively, in an antibody molecule.

The term “**monovalent**” as used herein with respect to a binding site of an antibody shall refer to a molecule comprising only one binding site directed against a target antigen. The term “valency” is thus understood as the number of binding sites directed against the same target antigen, either specifically binding the same or different epitopes of an antigen.

The antibody of the present invention is understood to comprise a monovalent, bivalent, tetravalent or multivalent binding site specifically binding oxMIF and HSG.

The term “**hypervariable region**” or “HVR,” as used herein refers to each of the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops (“hypervariable loops”). Generally, native four-chain antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). HVRs generally comprise amino acid residues from the hypervariable loops and/or from the “complementarity determining regions” (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition (Kabat et al., 1991) Hypervariable regions (HVRs) are also referred to as complementarity determining regions (CDRs),

and these terms are used herein interchangeably in reference to portions of the variable region that form the antigen binding regions. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

Kabat defined a numbering system for variable region sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of "Kabat numbering" to any variable region sequence, without reliance on any experimental data beyond the sequence itself. The Kabat numbering of residues can be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat et al., 1983, U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest". Unless otherwise specified, references to the numbering of specific amino acid residue positions in an antibody variable region are according to the Kabat numbering system. The numbering of the constant region is according to EU numbering index.

CDRs also comprise "specificity determining residues," or "SDRs," which are residues that contact an antigen. SDRs are contained within regions of the CDRs called abbreviated-CDRs, or a-CDRs. Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., *supra*. CDR determination can also be performed according to IMGT (Lefranc MP. 1997). IMGT has its own definitions of the framework regions (named FR-IMGT) and CDR (named CDR-IMGT). The IMGT numbering method counts residues continuously from 1 to 128 based on the germ-line V sequence alignment.

CDRs (or SDRs) can further be determined according to MacCallum RM et al., 1996. Herein, antigen-contacting residues are analyzed and site shape in the antibody Fv and Fab crystal structures available from the Protein Data Bank are combined. Antigen-contacting propensities are presented for each antibody residue, allowing a definition for CDRs to be proposed based on observed antigen contacts. Contacts are more common at CDR residues which are located centrally within the combining site; some less central CDR residues are only contacted by large antigens. Non-contacting residues within the CDRs coincide with residues identified by Chothia and co-workers (Chothia C et al., 1987) as important in defining "canonical" conformations.

A “**point mutation**” is particularly understood as the engineering of a polynucleotide that results in the expression of an amino acid sequence that differs from the non-engineered amino acid sequence in the substitution or exchange, deletion, or insertion of one or more single (non-consecutive) or doublets of amino acids for different amino acids. Preferred point mutations refer to the exchange of amino acids of the same polarity and/or charge.

“**Percent (%) sequence identity**” with respect to the polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific polypeptide sequence, after aligning the sequence and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

According to the present invention, sequence identity of the variable or constant region sequences is at least 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% with the respective sequences described herein.

As described above, the bispecific antibody described herein contains a first binding site for a tumor antigen or tumor-associated antigen and a second binding site for the HSG (histamine succinyl glycy) hapten, which specifically is located on a HSG moiety. In an alternative embodiment, the antibody can comprise two, three or more HSG hapten binding sites and one or more binding sites for a target antigen associated with a disease or condition.

Such HSG moieties can be of diverse structure and preferably comprise peptides having as few as two amino acid residues, more preferably two to ten residues, and may be coupled to other moieties, such as chelating agents. The HSG moiety preferably is a low molecular weight molecule, preferably having a molecular weight of less than 50,000 daltons, and advantageously less than about 20,000 daltons, 10,000 daltons or 5,000 daltons, including the chelates and chelated metals, and optionally a diagnostic and/or therapeutic agent. In a preferred embodiment, the HSG moiety has four or more residues, such as the peptide DOTA-Phe-Lys(HSG)-Tyr-Lys(HSG)-NH₂ (SEQ ID NO: 63), wherein DOTA is 1,4,7,10-tetraazacyclododecanetetraacetic acid. The HSG moiety may also comprise unnatural amino acids, e.g., D-amino acids, in the peptide backbone structure to increase the stability of the peptide in vivo.

Some specific embodiments of HSG moieties may include but are not limited to:

- DOTA-D-Asp-D-Lys(HSG)-D-Asp-D-Lys(HSG)-NH₂ (IMP 271; SEQ ID NO: 64)
- DOTA-D-Glu-D-Lys(HSG)-D-Glu-D-Lys(HSG)-NH₂ (IMP 277; SEQ ID NO: 65);
- DOTA-D-Tyr-D-Lys(HSG)-D-Glu-D-Lys(HSG)-NH₂ (IMP 288; SEQ ID NO: 66);
- 5 - DOTA-D-Ala-D-Lys(HSG)-D-Glu-D-Lys(HSG)-NH₂ (IMP 281; SEQ ID NO: 67);
- DOTA-D-Phe-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-NH₂ (IMP 284; SEQ ID NO: 68)
- DOTA-D-Lys(HSG)-D-Glu-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-NH₂ (IMP 301; SEQ ID NO: 69)
- [DOTA-D-Lys(HSG)-D-Ala-D-Lys(HSG)-D-Glu-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-
- 10 NH₂ (IMP 302; SEQ ID NO: 70)
- DOTA-D-Tyr-D-Lys(HSG)-D-Glu-D-Lys(HSG)-D-Cys-NH₂ (IMP 305; SEQ ID NO: 71)
- Ac-D-Lys(In-DTPA)-D-Tyr-D-Lys(In-DTPA)-D-Lys(Tscg-Cys)-NH₂ (IMP 297; SEQ ID NO: 72)
- 15 - HCO—CO-D-Lys(HSG)-D-Glu-D-Lys(HSG)-NH₂ (IMP 289; SEQ ID NO: 73);
- Ac-D-Phe-D-Lys(DOTA)-D-Tyr-D-Lys(DOTA)-NH₂ (SEQ ID NO: 74);
- Ac-D-Phe-D-Lys(DTPA)-D-Tyr-D-Lys(DTPA)-NH₂ (SEQ ID NO: 75);
- Ac-D-Phe-D-Lys(Bz-DTPA)-D-Tyr-D-Lys(Bz-DTPA)-NH₂ (SEQ ID NO: 76);
- Ac-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-D-Lys(Tscg-Cys)-NH₂ (SEQ ID NO: 77);
- 20 - DOTA-D-Phe-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-D-Lys(Tscg-Cys)-NH₂ (SEQ ID NO: 78);
- (Tscg-Cys)-D-Phe-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-D-Lys(DOTA)-NH₂ (SEQ ID NO: 79);
- Tscg-D-Cys-D-Glu-D-Lys(HSG)-D-Glu-D-Lys(HSG)-NH₂ (SEQ ID NO: 80);
- 25 - (Tscg-Cys)-D-Glu-D-Lys(HSG)-D-Glu-D-Lys(HSG)-NH₂ (SEQ ID NO: 81);
- Ac-D-Cys-D-Lys(DOTA)-D-Tyr-D-Ala-D-Lys(DOTA)-D-Cys-NH₂ (SEQ ID NO: 82);
- Ac-D-Cys-D-Lys(DTPA)-D-Tyr-D-Lys(DTPA)-NH₂ (SEQ ID NO: 83);
- Ac-D-Lys(DTPA)-D-Tyr-D-Lys(DTPA)-D-Lys(Tscg-Cys)-NH₂ (SEQ ID NO: 84);
- 30 and
- Ac-D-Lys(DOTA)-D-Tyr-D-Lys(DOTA)-D-Lys(Tscg-Cys)-NH₂ (SEQ ID NO: 85).

The peptides used as targetable constructs are conveniently synthesized on an automated peptide synthesizer using a solid-phase support and standard techniques of repetitive orthogonal deprotection and coupling. Free amino groups in the peptide, that

are to be used later for chelate conjugation, are advantageously blocked with standard protecting groups such as a Boc group, while N-terminal residues may be acetylated (Ac-) to increase serum stability. Such protecting groups will be known to the skilled artisan. See Greene and Wuts, *Protective Groups in Organic Synthesis*, 1999 (John Wiley and Sons, N.Y.). In preferred embodiments, the HSG moiety may comprise one or more hydrophilic chelate moieties, which can bind metal ions and can also help to ensure rapid in vivo clearance. Chelators may be selected for their particular metal-binding properties, and may be readily interchanged.

Particularly useful metal-chelate combinations include 2-benzyl-DTPA and its monomethyl and cyclohexyl analogs. Macrocyclic chelators such as NOTA (1,4,7-triazacyclononane-N,N',N''-triacetic acid), DOTA, TETA (p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid) and NETA are also of use with a variety of metals. DTPA and DOTA-type chelators, where the ligand includes hard base chelating functions such as carboxylate or amine groups, are most effective for chelating hard acid cations, especially Group IIa and Group IIIa metal cations. Such metal-chelate complexes can be made very stable by tailoring the ring size to the metal of interest. Other ring-type chelators such as macrocyclic polyethers are of interest for stably binding nuclides. Porphyrin chelators may be used with numerous metal complexes. More than one type of chelator may be conjugated to a carrier to bind multiple metal ions. Chelators such as thiosemicarbazonylglyoxylcysteine (Tscg-Cys) and thiosemicarbazinyl-acetylcysteine (Tsca-Cys) are advantageously used to bind soft acid cations of Tc, Re, Bi and other transition metals, lanthanides and actinides that are tightly bound to soft base ligands. It can be useful to link more than one type of chelator to a peptide. In a specific embodiment, two different hard acid or soft acid chelators can be incorporated into the targetable construct, e.g., with different chelate ring sizes, to bind preferentially to two different hard acid or soft acid cations, due to the differing sizes of the cations, the geometries of the chelate rings and the preferred complex ion structures of the cations. This will permit two different metals, to be incorporated into a HSG moiety for eventual capture by a bispecific antibody of the invention.

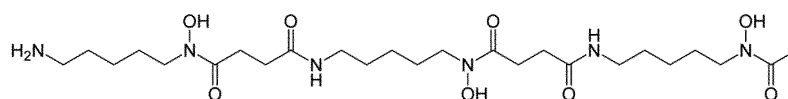
The HSG moiety can be labeled with or conjugated to one or more diagnostic and/or therapeutic agents.

Specifically, it can comprise one or more HSG haptens, one or more therapeutic/diagnostic agents, and one or more chelators.

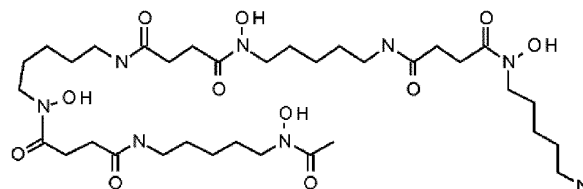
Some useful non-limiting examples are cyclic chelators or bifunctional chelating agents thereof like diethylenetriamine pentaacetic acid (DTPA), and 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetra acetic acid, ca-DTPA, ibca-DTPA, 1B4M-DTPA, lys-DTPA, vinyl DTPA, glu-DTPA, p-SCN-bn-DOTA, DOTA-NHS-ester, deferoxamine B or derivatives thereof; or linear chelators or bifunctional chelating agents thereof like p-SCN-Bn-DTPA, HOPO and CHX-A"-DTPA, ethylenediamine tetraacetic acid (EDTA), DTPA, EDTMP, NOTA, TETA, DOTMP, N2S2, N3S, HEDP. Examples of further suitable chelating agents include DOTA derivatives such as p-isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bz-DOTA) and DTPA derivatives such as p-isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (p-SCN-Bz-DTPA), the first being cyclic chelators, the latter being linear chelators.

Further examples of deferoxamine (desferrioxamine, DFO) and derivatives thereof include, but are not limited to p-NCS-Bz-DFO, DFOSq, DFO*, oxoDFO*, DFO*Sq, DFO*-NCS, DFO*pPhe-NCS.

Specifically, the DFO can be of following structure:



Specifically, DFO* can be of following structure:



The peptide backbone and DOTA may be linked together either directly or via a cleavable or non-cleavable linker.

In certain embodiments, the HSG moiety disclosed herein can be attached to one or more therapeutic and/or diagnostic agents. Therapeutic agents are specifically selected from the group consisting of a radionuclide, an anti-angiogenic agent, a drug, a prodrug, a pro-apoptotic agent, an interference RNA, a photoactive therapeutic agent, a cytotoxic agent, which may be a chemotherapeutic agent or a toxin, and a combination thereof. The drugs of use may possess a pharmaceutical property selected from the

group consisting of antimetabolic, anti-kinase, alkylating, anti-metabolite, antibiotic, alkaloid, anti-angiogenic, pro-apoptotic agents, and combinations thereof.

In a preferred embodiment the of cytotoxic drugs are selected from, but limited to, microtubule inhibitors, meiosis inhibitors, RNA polymerase inhibitors, topoisomerase
5 inhibitors, DNA damaging agents, and ribosome inhibitors.

Microtubule inhibitors or tubulin inhibitors, such as auristatins block tubulin assembly and cause G2/M phase cell cycle arrest. Monomethyl auristatin F and monomethyl auristatin E, auristatin derivatives (MMAF, MMAE) are effective in the low nanomolar range (Gerber et al, 2009). Maytansinoids or maytansine derivatives are
10 another class of tubulin inhibitors, such as emtansine (DM1) and ravtansine (DM4). Tubulysins and analogues thereof are a further class of tubulin inhibitors. Microtubule inhibitors (MTI) such as taxanes, vinca alkaloids, and epothilones stabilize or destabilize microtubules, thereby suppressing microtubule dynamics required for proper mitotic function, effectively blocking cell cycle progression, and resulting in apoptosis (Perez E.,
15 2009).

Meiosis inhibitors can be for example cyclin-dependent kinase 2 (CDK2) such as flavopiridol, CY-202 (Malumbres M. et al.2008). The DNA-damaging agents have the ability to be active throughout the different cell cycle phases. Duocarmycin is a cytotoxic DNA-alkylating compound that binds to the minor groove of DNA. Anthracyclines and
20 analogues such as carminomycin, daunorubicin and doxorubicin are DNA-intercalating compounds which can be used as drug conjugates. Calicheamicin is a potent antitumor antibiotic that causes double-strand DNA breaks and rapid cell death by binding to the DNA's minor groove. It is less dependent on cell cycle progression making it potentially useful against TICs who have lower rates of proliferation (Gupta et al, 2009, Sapra et al,
25 2011). As an alternative, ozogamycin can be used. Another new category of DNA-damaging agents are pyrrolobenzodiazepines (PBDs) that bind to discrete DNA sequences causing lethal lesions.

A potential new drug under investigation is α -amanitin, an RNA polymerase II inhibitor in the picomolar range, derived from the mushroom, *Amanita phalloides*
30 (Moldenhauer et al, 2012).

Other exemplary drugs of use include, but are not limited to, 5-fluorouracil, aplidin, azaribine, anastrozole, anthracyclines, bendamustine, bleomycin, bortezomib, bryostatin-1, busulfan, calicheamicin, camptothecin, carboplatin, 10-hydroxycamptothecin, carmustine, CELEBREX® (celecoxib), chlorambucil, cisplatin

(CDDP), Cox-2 inhibitors, irinotecan (CPT-11), SN-38, carboplatin, cladribine, camptothecans, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunorubicin, doxorubicin, 2-pyrrolinodoxorubicine (2P-DOX), cyano-morpholino doxorubicin, doxorubicin glucuronide, epirubicin glucuronide, estramustine, 5 epipodophyllotoxin, estrogen receptor binding agents, etoposide (VP16), etoposide glucuronide, etoposide phosphate, floxuridine (FUdR), 3',5'-O-dioleoyl-FudR (FUdR-dO), fludarabine, flutamide, farnesyl-protein transferase inhibitors, gemcitabine, hydroxyurea, idarubicin, ifosfamide, L-asparaginase, lenolidamide, leucovorin, lomustine, mechlorethamine, melphalan, mercaptopurine, 6-mercaptopurine, 10 methotrexate, mitoxantrone, mithramycin, mitomycin, mitotane, navelbine, nitrosourea, plicomycin, procarbazine, paclitaxel, pentostatin, PSI-341, raloxifene, semustine, streptozocin, tamoxifen, Taxol®, temazolomide (an aqueous form of DTIC), transplatinum, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinorelbine, vinblastine, vincristine and *vinca* alkaloids.

15 Toxins of use may include ricin, abrin, alpha toxin, saporin, ribonuclease (RNase), e.g., onconase, DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtheria toxin, *Pseudomonas* exotoxin, and *Pseudomonas* endotoxin.

Radioactive isotopes (radioisotopes, radionuclides) useful for treating diseased tissue include, but are not limited to ^{11}C , ^{13}N , ^{15}O , ^{18}F , ^{32}P , ^{33}P , ^{47}Sc , ^{59}Fe , ^{64}Cu , ^{67}Cu , 20 ^{67}Ga , ^{75}Se , ^{77}As , ^{89}Sr , ^{89}Zr , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{99}Mo , ^{103}Pd , ^{105}Rh , ^{109}Pd , ^{111}Ag , ^{111}In , ^{123}I , ^{124}I , ^{125}I , ^{131}I , ^{140}La , ^{142}Pr , ^{143}Pr , ^{149}Tb , ^{149}Pm , ^{153}Sm , ^{159}Gd , ^{161}Tb , ^{165}Dy , ^{166}Dy , ^{166}Ho , ^{169}Yb , ^{169}Er , ^{175}Yb , ^{177}Lu , ^{186}Re , ^{188}Re , ^{189}Re , ^{192}Ir , $^{193\text{m}}\text{Pt}$, $^{195\text{m}}\text{Pt}$, ^{194}Ir , ^{198}Au , ^{199}Au , ^{211}At , ^{211}Pb , ^{212}Pb , ^{212}Bi , ^{213}Bi , ^{211}At , ^{223}Ra , ^{225}Ac , and ^{227}Th .

Particularly useful therapeutic radionuclides include, but are not limited to, ^{32}P , 25 ^{33}P , ^{47}Sc , ^{64}Cu , ^{67}Cu , ^{67}Ga , ^{90}Y , ^{111}Ag , ^{111}In , ^{125}I , ^{131}I , ^{142}Pr , ^{153}Sm , ^{161}Tb , ^{166}Dy , ^{166}Ho , ^{177}Lu , ^{186}Re , ^{188}Re , ^{189}Re , ^{212}Pb , ^{212}Bi , ^{213}Bi , ^{211}At , ^{223}Ra and ^{225}Ac .

Particularly useful diagnostic/detection radionuclides include, but are not limited to, ^{18}F , ^{52}Fe , ^{62}Cu , ^{64}Cu , ^{67}Cu , ^{67}Ga , ^{68}Ga , ^{86}Y , ^{89}Zr , $^{94\text{m}}\text{Tc}$, ^{94}Tc , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{123}I , ^{124}I , 30 ^{125}I , ^{131}I , $^{154-158}\text{Gd}$, ^{32}P , ^{90}Y , ^{188}Re , and ^{175}Lu .

The therapeutic radionuclide preferably has a decay-energy in the range of 20 to 6,000 keV, preferably in the ranges 60 to 200 keV for an Auger emitter, 100-2,500 keV for a beta emitter, and 4,000-6,000 keV for an alpha emitter. Maximum decay energies of useful beta-particle-emitting nuclides are preferably 20-5,000 keV, more preferably 100-4,000 keV, and most preferably 500-2,500 keV. Also preferred are radionuclides

that substantially decay with Auger-emitting particles. For example, ^{58}Co , ^{67}Ga , $^{80\text{m}}\text{Br}$, $^{99\text{m}}\text{Tc}$, $^{103\text{m}}\text{Rh}$, ^{109}Pt , ^{111}In , ^{119}Sb , ^{125}I , ^{161}Ho , ^{189}mOs and ^{192}Ir . Decay energies of useful beta-particle-emitting nuclides are preferably <1,000 keV, more preferably <100 keV, and most preferably <70 keV. Also preferred are radionuclides that substantially decay with generation of alpha-particles. Such radionuclides include, but are not limited to: ^{152}Dy , ^{211}At , ^{212}Bi , ^{223}Ra , ^{219}Rn , ^{215}Po , ^{211}Bi , ^{225}Ac , ^{221}Fr , ^{217}At , ^{213}Bi and ^{255}Fm . Decay energies of useful alpha-particle-emitting radionuclides are preferably 2,000-10,000 keV, more preferably 3,000-8,000 keV, and most preferably 4,000-7,000 keV.

Specifically, the radionuclide is selected from the group consisting of ^{67}Ga , ^{89}Zr , ^{111}In , ^{124}I , ^{131}I , ^{177}Lu , and ^{225}Ac .

Therapeutic agents may also include a photoactive agent or a dye. Fluorescent compositions, such as fluorochrome, and other chromogens, or dyes, such as porphyrins sensitive to visible light, have been used to detect and to treat lesions by directing the suitable light to the lesion. In therapy, this has been termed photoradiation, phototherapy, or photodynamic therapy.

In certain embodiments, anti-angiogenic agents, such as angiostatin, baculostatin, canstatin, maspin, tissue metalloproteinase inhibitors, 2-methoxyoestradiol, carboxyamidotriazole, CM101, Marimastat, pentosan polysulphate, herbimycin A, PNU145156E, Linomide, thalidomide, pentoxifylline, genistein, TNP-470, endostatin, paclitaxel, accutin, angiostatin, cidofovir, vincristine, bleomycin, AGM-1470, or minocycline may be of use.

The therapeutic agent may comprise an oligonucleotide, such as a siRNA. The skilled artisan will realize that any siRNA or interference RNA species may be attached to a targetable construct for delivery to a targeted tissue. Many siRNA species against a wide variety of targets are known in the art, and any such known siRNA may be utilized in the claimed methods and compositions.

Known siRNA species of potential use include those specific for IKK-gamma, VEGF, Flt-1, and Flk-1/KDR, Bcl2 and EGFR, CDC20, transducin (beta)-like 3; KRAS; carbonic anhydrase II; complement component 3; interleukin-1 receptor-associated kinase 4 (IRAK4); survivin; superoxide dismutase 1; MET proto-oncogene; amyloid beta precursor protein (APP); IGF-1R; ICAM1; complement factor B; p53, and apolipoprotein B.

Diagnostic agents are preferably selected from the group consisting of a radionuclide, a radiological contrast agent, a paramagnetic ion, a metal, a fluorescent

label, a chemiluminescent label, an ultrasound contrast agent, and a photoactive agent. Such diagnostic agents are well known and any such known diagnostic agent may be used. Non-limiting examples of diagnostic agents may include a radionuclide such as ^{18}F , ^{52}Fe , ^{110}In , ^{111}In , ^{177}Lu , ^{52}Fe , ^{62}Cu , ^{64}Cu , ^{67}Cu , ^{67}Ga , ^{68}Ga , ^{86}Y , ^{90}Y , ^{89}Zr , $^{94\text{m}}\text{Tc}$, ^{94}Tc ,
5 $^{99\text{m}}\text{Tc}$, ^{120}I , ^{123}I , ^{124}I , ^{125}I , ^{131}I , $^{154-158}\text{Gd}$, ^{32}P , ^{11}C , ^{13}N , ^{15}O , ^{186}Re , ^{188}Re , ^{51}Mn , $^{52\text{m}}\text{Mn}$, ^{55}Co , ^{72}As , ^{75}Br , ^{76}Br , $^{82\text{m}}\text{Rb}$, ^{83}Sr , or other gamma-, beta-, or positron-emitters.

The three most commonly used PET radionuclides are ^{18}F , ^{68}Ga and ^{89}Zr .

Synthetic peptides may also be useful herein. E.g., IMP-288 and IMP-449 are Tyr-D-Lys-D-Glu-D-Lys (SEQ ID NO: 86) tetrapeptides in which both lysine residues are
10 substituted with a HSG-moiety via their ϵ -amino group. IMP-449 is conjugated with NOTA: 1,4,7-tri-azacyclononane-N,N',N''-triacetic acid.

IMP-288 is a DOTA-conjugated D-Tyr-D-Lys-D-Glu-D-Lys-NH₂ (SEQ ID NO: 87) tetrapeptide in which both lysine residues are derivatized with HSG moiety via their ϵ -aminogroup.

15 IMP-453, IMP402, IMP457, or IMP498 as described in US9352036B2 may also be used.

Paramagnetic ions of use may include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

20 Metal contrast agents may include lanthanum (III), gold (III), lead (II) or bismuth (III).

Ultrasound contrast agents may comprise liposomes, such as gas filled liposomes. Radiopaque diagnostic agents may be selected from compounds, barium compounds, gallium compounds, and thallium compounds. A wide variety of fluorescent labels are known in the art, including but not limited to fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and
25 fluorescamine. Chemiluminescent labels of use may include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt, europium(III), or an oxalate ester.

The HSG moieties can be of diverse structure and are selected not only for the
30 availability of an antibody or fragment that binds with high affinity to the HSG moiety, but also for rapid in vivo clearance when used within the pre-targeting method. Hydrophobic agents are best at eliciting strong immune responses, whereas hydrophilic agents are preferred for rapid in vivo clearance. Thus, a balance between hydrophobic and hydrophilic character is established. This may be accomplished, in part, by using

hydrophilic chelating agents to offset the inherent hydrophobicity of many organic moieties. Also, subunits of the targetable construct may be chosen which have opposite solution properties, for example, peptides, which contain amino acids, some of which are hydrophobic and some of which are hydrophilic. Aside from peptides, carbohydrates
5 may also be used.

Pre-targeting is a multistep process originally developed to resolve the slow blood clearance of directly targeting antibodies, which contributes to undesirable toxicity to normal tissues. With pre-targeting, a diagnostic or therapeutic agent, such as a radionuclide, is attached to a small delivery molecule that is cleared within minutes from
10 the blood. According to an embodiment of the invention, the small delivery molecule is a HSG moiety described herein. The pre-targeting bispecific antibody of the invention, which has one or more binding sites for the HSG moiety as well as a target tumor antigen, is administered first, free antibody is allowed to clear from circulation and then the targetable construct is administered.

15 A pre-targeting method of treating or diagnosing a disease or disorder in a subject may be provided by: (1) administering to the subject a bispecific antibody; (2) optionally administering to the subject a clearing composition, and allowing the composition to clear the antibody from circulation; and (3) administering to the subject the HSG moiety described herein, containing one or more chelated or chemically bound therapeutic or
20 diagnostic agents.

The bispecific antibodies described herein may be used in the treatment or detection of malignancies in a pre-targeting method, wherein the antibody may be administered first to a subject. Sufficient time may be allowed for the bispecific antibody to bind to a target antigen and for unbound antibody to clear from circulation.

25 Optionally, a clearing composition may be administered after administering the antibody, allowing the clearing composition to remove the antibody from circulation. Clearing compositions can be, but are not limited to avidin, galactose, antibodies against the pretargeting antibody, and Fc-antigen fusions. ABDEG (Antibody that enhances IgG degradation) clearing approach includes administration of antigen-specific IgG antibody
30 followed by, after the antigen-specific mAb is localised in the tumor, irrelevant IgG-YTE (=antibody with enhanced binding to FcRn via its M252Y/S254T/T256E mutations in the Fc) which through its stronger binding to FcRn enhances degradation of the free antigen-specific antibody/its clearance from circulation (Nazarova L. et al, 2020).

Then a targetable construct, such as the HSG moiety described above, labeled with, or linked to a diagnostic agent such as a radionuclide, may be administered to the subject and allowed to bind to the bispecific antibody and localize at the diseased cell or tissue.

5 Slow blood clearance and delayed tumor uptake of directly radiolabelled antibodies in solid tumors cause continuous high radiation dose exposure to healthy tissues and organs. *In vivo* pre-targeted radioimmunotherapy (PRAIT) using the antibodies of the invention allows to overcome these limitations. PRAIT aims at improving the therapeutic index (tumor-to-normal tissue ratios) by delivering increased
10 absorbed doses to tumors, as compared to directly radiolabeled antibodies or antibody fragments. PRAIT involves the administering a the herein described bispecific antibodies recognizing HSG and a tumor target, followed administration of a radiolabeled HSG hapten moiety. The administration of the radiolabeled HSG hapten moiety can be a few days later, e.g. 2, 3, 4, 5, 6, or 7 days later. With this technology, a substantial amount
15 of the inventive antibody is accumulated in or on the surface of the tumor or on the surface of malignant cells and is cleared from the circulation to a large extend and the radioactive labeled HSG hapten moiety binds to the antibodies accumulated in the tumor or on the surface of the tumor or on the surface of malignant cells, whereas the non-bound radioactive HSG hapten clears from the circulation through the kidneys within a
20 few hours. Thus, resulting in minimizing the exposure of normal organs to radioactivity.

For targeted radionuclide therapy, e.g. HSG hapten IMP-288 (as described in US2005/0025709) and ¹⁷⁷Lu as radionuclide may be used.

For treatment purposes, also the pre-targeting approach can be used as described above. In this case, the HSG moiety comprises one or more of the therapeutic
25 agents described above. Specifically, the HSG hapten IMP-453 may be used, directly coupled to the therapeutic agent.

A “**subject**” is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain
30 embodiments, the individual or subject is a human.

According to the invention, the antibody described herein may be used for preparing a medicament.

Also, a pharmaceutical composition containing the antibody of the invention is encompassed herein.

The term “**pharmaceutical composition**” refers to a preparation together with a pharmaceutical excipient, such as a carrier, which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which
5 the formulation would be administered.

A “**pharmaceutically acceptable carrier**” refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. Some examples of pharmaceutically acceptable carriers are water, saline, phosphate buffered saline, amino acids such as glycine or histidine, dextrose, glycerol,
10 ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Additional examples of pharmaceutically acceptable substances are wetting agents or minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives, or buffers, which enhance the shelf
15 life or effectiveness of the antibody.

As used herein, “**treatment**”, “treat” or “treating” refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence
20 of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

25 The bispecific anti-tumor antigen/anti-HSG antibody of the invention and the pharmaceutical compositions comprising it, can be administered to a subject in a first step and a HSG moiety is administered in a second step, wherein said HSG moiety binds to the antibody.

The pharmaceutical compositions of this invention may be in a variety of forms,
30 for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, and powders. The preferred form depends on the intended mode of administration and therapeutic or diagnostic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive

immunization of humans. The preferred mode of administration is parenteral (e.g., intravenous, intraarterial, intralymphatic or intrathecal). In a preferred embodiment, the antibody is administered by intravenous infusion or injection. In another preferred embodiment, the antibody is administered by intravenous injection. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

The administration of the bispecific anti-tumor antigen/anti-HSG antibody of the invention and the labeled or conjugated HSG moiety may be conducted by administering the bsAb antibody at some time prior to administration of the labeled or conjugated HSG moiety. The doses and timing of the reagents can be readily devised by a skilled artisan, and are dependent on the specific nature of the reagents employed. In a specific embodiment the bispecific anti-tumor antigen/anti-HSG antibody is given first, then after sufficient time has passed for the bsAb of the invention to target to the diseased tissue, specifically 24-72 hours, or in an alternative embodiment 48-96 hours, the labeled or conjugated HSG moiety is administered. When the bispecific anti-tumor antigen/anti-HSG antibody of the invention is administered, specifically in a pretargeting technique, the dosage of an administered antibody for humans will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Typically, it is desirable to provide the recipient with a dosage of bispecific antibody that is in the range of from about 1 mg to 400 mg as a single intravenous infusion, although a lower or higher dosage also may be administered as circumstances dictate. Typically, it is desirable to provide the recipient with a dosage 1 to 200 mg, more preferably 1 to 70 mg, most preferably 1 to 20 mg, although higher or lower doses may be used. Dosages of therapeutic bispecific antibodies may be higher, such as 1 to 200, 1 to 100, 100 to 1000, 100 to 500, 200 to 750 mg. The dose for each subject depends on the conditions, such as weight or type of disease. However, the dosage can be determined by the skilled person with reference to general knowledge.

In general, the dosage of labeled or conjugated HSG moieties to administer will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, and previous medical history. Preferably, a saturating dose of the labeled or conjugated HSG moieties is administered to a patient. For administration of radiolabeled molecules, the dosage may be measured by millicuries.

The anti-tumor antigen/anti-HSG antibody described herein may be administered once, or, in an alternative embodiment, multiple times each followed by a sequential

administration of a labeled or conjugated HSG moiety for diagnosing and/or therapy of malignancies.

In preferred embodiments, anti-tumor antigen/anti-HSG antibody of the invention are of use for therapy of cancer.

5 The term “**cancer**” as used herein refers to malignancies, specifically to proliferative diseases, specifically to solid cancers, such as colorectal cancer, ovarian cancer, pancreas cancer, lung cancer, melanoma, squamous cell carcinoma (SCC) (e.g., head and neck, esophageal, and oral cavity), hepatocellular carcinoma, colorectal adenocarcinoma, kidney cancer, medullary thyroid cancer, papillary thyroid cancer,
10 astrocytic tumor, neuroblastoma, Ewing’s sarcoma, non-Hodgkin’s lymphomas, B-cell acute and chronic lymphoid leukemias, Burkitt lymphoma, Hodgkin’s lymphoma, hairy cell leukemia, acute and chronic myeloid leukemias, T-cell lymphomas and leukemias, multiple myeloma, glioma, Waldenstrom’s macroglobulinemia, melanomas, sarcomas, gliomas, skin cancers, cervical cancers, endometrial carcinoma, breast cancers,
15 prostate cancers, gastric cancers, and malignant seminoma, including refractory versions of any of the above cancers, or a combination of one or more of the above cancers.

 Hyperproliferative disorders, such as cancerous diseases or cancer, that may be treated or detected by the anti-tumor antigen/anti-HSG antibodies of the invention can
20 involve any tissue or organ and include but are not limited to brain, squamous cell, bladder, head, neck, liver, ovarian, esophageal, nasopharynx, or thyroid cancers, melanomas, lymphomas, leukemias or multiple myelomas, oral cavity, gastrointestinal tract, colon, colorectal, stomach, pancreas, pulmonary tract, lung, breast, ovary, prostate, uterus, endometrium, cervix, urinary bladder, pancreas, bone, liver, gall
25 bladder, kidney, skin, and testes.

 In particular, the anti-tumor antigen/anti-HSG antibodies of the invention are useful to treat hematological and solid tumors such as colorectal cancer, ovarian cancer, breast cancer, prostate cancer, pancreas cancer, and lung cancer.

 In a specific embodiment, the antibodies highly suitable for the treatment of
30 cancerous diseases, specifically for the treatment of solid tumors are bispecific anti-tumor antigen/anti-HSG antibodies comprising a binding site specifically recognizing HSG, comprising

 - a light chain variable (VL) domain comprising SEQ ID NO: 17 or SEQ ID NO: 19, or a sequence having at least 95% identity to SEQ ID NOs: 17 or 19, specifically with

glutamine at position 100 (Q100) and isoleucine at position 106 (I106) according to Kabat numbering and

- a heavy chain variable (VH) domain: comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10 or a sequence having at least 95% identity to SEQ ID NOs: 4, 6, 8, and 10, specifically with arginine at position 19 (R19) according to Kabat numbering, and

- optionally said VL and VH domains are connected via a linker sequence, and a binding site specifically recognizing a tumor antigen comprising
 - (a1) a light chain variable domain comprising SEQ ID NO: 45 with one or more amino acid substitutions M30L, F49Y, A51G, P80S, W93F, or
 - (a2) a light chain variable domain having at least 95% sequence identity to SEQ ID NO: 45 having a conserved tyrosine at position 36, and one or more amino acid substitutions M30L, F49Y, A51G, P80S, W93F;

and

- (b1) a heavy chain variable domain comprising SEQ ID NO: 46 or
- (b2) a heavy chain variable domain comprising SEQ ID NO: 46 with amino acid substitutions L5Q and/or W97Y, or
- (b3) a heavy chain variable domain having at least 95% sequence identity to SEQ ID NO: 46 having amino acid substitutions L5Q and/or W97Y,

wherein amino acid positions are numbered according to Kabat.

In a further specific embodiment, the antibodies highly suitable for the treatment of cancerous diseases, specifically for the treatment of solid tumors are bispecific anti-tumor antigen/anti-HSG antibodies comprising a binding site specifically recognizing HSG, comprising

- a light chain variable (VL) domain comprising SEQ ID NO: 17 or SEQ ID NO: 19, or a sequence having at least 95% identity to SEQ ID NOs: 17 or 19, specifically with glutamine at position 100 (Q100) and isoleucine at position 106 (I106) according to Kabat numbering and

- a heavy chain variable (VH) domain: comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10 or a sequence having at least 95% identity to SEQ ID NOs: 4, 6, 8, and 10, specifically with arginine at position 19 (R19), and

- optionally said VL and VH domains are connected via a linker sequence, and a binding site specifically recognizing a tumor antigen comprising

- a light chain variable domain comprising SEQ ID NO: 27, or a light chain variable domain comprising SEQ ID NO: 27 and further comprising amino acid substitutions M30L and/or P80S, and

5 - a heavy chain variable domain comprising SEQ ID NO: 46, specifically with amino acid substitutions L5Q and/or W97Y,

wherein the amino acid positions are numbered according to Kabat.

In a further specific embodiment, the antibodies are bispecific anti-tumor antigen/anti-HSG antibodies comprising a binding site specifically recognizing HSG, comprising

10 - a light chain variable (VL) domain comprising any one of SEQ ID NOs: 17, 19, 89, 90 and 91, and

a heavy chain variable (VH) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10.

15 In yet an alternative embodiment, the antibodies are bispecific anti-tumor antigen/anti-HSG antibodies comprising a binding site specifically recognizing HSG, further comprising

- a light chain variable (VL) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 17, 19, 89, 90, and 91, and

20 - a heavy chain variable (VH) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10 with one or two further amino acid substitutions and with arginine at position 19 (R19) according to Kabat numbering.

In yet an alternative embodiment, the antibodies are bispecific anti-tumor antigen/anti-HSG antibodies comprising a binding site specifically recognizing HSG, further comprising

25 - a light chain variable (VL) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 17, 19, 89, 90, and 91 with one or two further amino acid substitutions and with glutamic acid at position 105 (E105) and isoleucine at position 106 (I106) according to Kabat numbering, and

30 - a heavy chain variable (VH) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10.

In yet an alternative embodiment, the antibodies are bispecific anti-tumor antigen/anti-HSG antibodies comprising a binding site specifically recognizing HSG, further comprising

- a light chain variable (VL) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 17, 19, 89, 90, and 91, and further 1 or 2 amino acid substitutions, with the proviso that glutamic acid at position 105 (E105) and isoleucine at position 106 (I106) are preserved, and

- 5 - a heavy chain variable (VH) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10 with one or two further amino acid substitutions and with arginine at position 19 (R19) according to Kabat numbering.

Optionally, the sequence also comprises glutamine at position 100 (Q100) to Kabat numbering.

- 10 Also provided herein is a nucleic acid encoding the antibody of the invention.

An **“isolated” nucleic acid** refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally, or at a chromosomal location that is different from its natural chromosomal location.

- 15 **“Isolated nucleic acid encoding an anti-tumor antigen/anti HSG antibody”** refers to one or more nucleic acid molecules encoding antibody heavy and light chains (or fragments thereof), including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in
20 a host cell.

- “No substantial cross-reactivity”** means that a molecule (e.g., an antibody) does not recognize or specifically bind an antigen different from the actual target antigen of the molecule (e.g. an antigen closely related to the target antigen), specifically reduced MIF, particularly when compared to that target antigen. For example, an
25 antibody may bind less than about 10% to less than about 5% to an antigen different from the actual target antigen, or may bind said antigen different from the actual target antigen at an amount consisting of less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.2%, or 0.1%, preferably less than about 2%, 1%, or 0.5%, and most preferably less than about 0.2% or 0.1% to an antigen different from the actual target
30 antigen. Binding can be determined by any method known in the art such as, but not limited to ELISA or surface plasmon resonance.

The recombinant production of the antibody of the invention preferably employs an expression system, e.g. including expression constructs or vectors comprising a nucleotide sequence encoding the antibody format.

The term “**expression system**” refers to nucleic acid molecules containing a desired coding sequence and control sequences in operable linkage, so that hosts, transformed or transfected with these sequences, are capable of producing the encoded proteins. In order to effect transformation, the expression system may be included on a
5 vector; however, the relevant DNA may then also be integrated into the host chromosome. Alternatively, an expression system can be used for *in vitro* transcription/translation.

“**Expression vectors**” used herein are defined as DNA sequences that are required for the transcription of cloned recombinant nucleotide sequences, i.e. of
10 recombinant genes and the translation of their mRNA in a suitable host organism. Expression vectors comprise the expression cassette and additionally usually comprise an origin for autonomous replication in the host cells or a genome integration site, one or more selectable markers (e.g. an amino acid synthesis gene or a gene conferring resistance to antibiotics such as zeocin, kanamycin, G418 or hygromycin), a number of
15 restriction enzyme cleavage sites, a suitable promoter sequence and a transcription terminator, which components are operably linked together. The terms “plasmid” and “vector” as used herein include autonomously replicating nucleotide sequences as well as genome integrating nucleotide sequences.

Specifically, the term refers to a vehicle by which a DNA or RNA sequence (e.g.
20 a foreign gene), e.g. a nucleotide sequence encoding the antibody format of the present invention, can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence. Plasmids are preferred vectors of the invention.

Vectors typically comprise the DNA of a transmissible agent, into which foreign
25 DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites.

A “**cassette**” refers to a DNA coding sequence or segment of DNA that code for an expression product that can be inserted into a vector at defined restriction sites. The
30 cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a “DNA construct”. A common type of vector is a

“plasmid”, which generally is a self-contained molecule of double-stranded DNA that can readily accept additional (foreign) DNA and which can readily be introduced into a suitable host cell. A vector of the invention often contains coding DNA and expression control sequences, e.g. promoter DNA, and has one or more restriction sites suitable for inserting foreign DNA. Coding DNA is a DNA sequence that encodes a particular amino acid sequence for a particular polypeptide or protein such as an antibody format of the invention. Promoter DNA is a DNA sequence which initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. Promoter DNA and coding DNA may be from the same gene or from different genes, and may be from the same or different organisms. Recombinant cloning vectors of the invention will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes.

The procedures used to ligate DNA sequences, e.g. providing or coding for the factors of the present invention and/or the protein of interest, a promoter, a terminator and further sequences, respectively, and to insert them into suitable vectors containing the information necessary for integration or host replication, are well known to persons skilled in the art, e.g. described by Sambrook et al, 2012.

Also encompassed herein is the production of the inventive antibody using a host cell.

A **host cell** is specifically understood as a cell, a recombinant cell or cell line transfected with an expression construct, such as a vector according to the invention.

The term “**host cell line**” as used herein refers to an established clone of a particular cell type that has acquired the ability to proliferate over a prolonged period of time. The term host cell line refers to a cell line as used for expressing an endogenous or recombinant gene to produce polypeptides, such as the recombinant antibody format of the invention.

A “**production host cell**” or “production cell” is commonly understood to be a cell line or culture of cells ready-to-use for cultivation in a bioreactor to obtain the product of a production process, the recombinant antibody format of the invention. The host cell type according to the present invention may be any prokaryotic or eukaryotic cell.

The term “**recombinant**” as used herein shall mean “being prepared by genetic engineering” or “the result of genetic engineering”, e.g. specifically employing heterologous sequences incorporated in a recombinant vector or recombinant host cell.

An antibody of the invention may be produced using any known and well-established expression system and recombinant cell culturing technology, for example, by expression in bacterial hosts (prokaryotic systems), or eukaryotic systems such as yeasts, fungi, insect cells or mammalian cells. An antibody molecule of the present invention may be produced in transgenic organisms such as a goat, a plant or a transgenic mouse, an engineered mouse strain that has large fragments of the human immunoglobulin loci and is deficient in mouse antibody production. An antibody may also be produced by chemical synthesis.

According to a specific embodiment, the host cell is a production cell line of cells selected from the group consisting of CHO, PerC6, CAP, HEK, HeLa, NS0, SP2/0 hybridoma and Jurkat. More specifically, the host cell is obtained from CHO cells.

The host cell of the invention is specifically cultivated or maintained in a serum-free culture, e.g. comprising other components, such as plasma proteins, hormones, and growth factors, as an alternative to serum.

Host cells are most preferred, when being established, adapted, and completely cultivated under serum free conditions, and optionally in media which are free of any protein/peptide of animal origin.

Anti-tumor antigen/anti-HSG antibodies of the invention can be recovered from the culture medium using standard protein purification methods.

The invention further encompasses following embodiments:

1. A bispecific anti-tumor antigen/anti-HSG antibody, comprising a binding site specifically recognizing a tumor antigen and a binding site specifically recognizing HSG, comprising
 - a light chain variable (VL) domain comprising SEQ ID NO: 17 or SEQ ID NO: 19, or a sequence having at least 95% identity to SEQ ID NOs: 17 or 19, with glutamine at position 100 (Q100) and isoleucine at position 106 (I106) according to Kabat numbering, and
 - a heavy chain variable (VH) domain: comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10, or a sequence having at least 95% identity to SEQ ID NOs: 4, 6, 8, and 10, with arginine at position 19 (R19) according to Kabat numbering.
2. The antibody of embodiment 1, comprising a single-chain variable fragment (scFv) specifically recognizing HSG of the formula selected from the group consisting of

VH-linker-VL-linker, VL-linker -VH-linker, wherein the linker comprises SEQ ID NO: 39 or SEQ ID NO: 42.

3. The antibody of embodiment 1, comprising VL and VH domains specifically recognizing HSG, wherein the VL domain is C-terminally connected to a CH1cross
5 comprising SEQ ID NO: 30, and the VH domain is C-terminally connected to a CLcross comprising SEQ ID NO: 38.

4. The antibody of any one of embodiments 1 to 3, comprising a Fc region having SEQ ID 31 comprising asymmetric mutations in each CH3 domain enabling heterodimerization of two CHs from different antibodies, specifically one CH comprises
10 „knob“ mutations T366W and S354C and one CH comprises „hole“ mutations T366S, L368A, Y407V and Y349C according to EU numbering index.

5. The antibody of any one of embodiments 1 to 4, comprising a variant Fc region with reduced or eliminated effector functions and/or FcRn binding.

6. The antibody of any one of embodiments 1 to 5, comprising a variant Fc
15 region having amino acid substitutions at any one or more of positions E233, L234, L235, G236, G237, P238, I253, D265, S267, H268, N297, S298, T299, H310, E318, L328, P329, A330, P331, H435 of SEQ ID NO: 31 according to EU numbering index, and optionally an aglycosylated Fc region.

7. The antibody of any one of embodiments 1 to 3, comprising two constant
20 heavy chain domains having a hinge and a Fc domain comprising SEQ ID NO: 35 and SEQ ID NO: 32.

8. The antibody of any one of embodiments 1 to 3, comprising two constant heavy chain regions having a hinge and a Fc domain comprising SEQ ID NO: 36 and
SEQ ID NO: 33.

25 9. The antibody of any one of embodiments 1 to 8, wherein the anti-tumor antigen is selected from the group consisting of oxMIF, Mesothelin (MSLN), and Folate Receptor alpha (FR α).

10. The antibody of any one of embodiments 1 to 9, wherein the anti-tumor antigen is oxMIF.

30 11. The antibody of embodiment 10, wherein the binding site specifically recognizing oxMIF comprises,

- a light chain variable domain comprising SEQ ID NO: 27, or a light chain variable domain comprising SEQ ID NO: 27 and further comprising amino acid substitutions M30L and/or P80S, and

- a heavy chain variable domain comprising SEQ ID NO: 46, specifically with amino acid substitutions L5Q and/or W97Y,

wherein the amino acid positions are numbered according to Kabat.

12. The antibody of any one of embodiments 1 to 11, selected from the group
5 consisting of Fab-scFv-Fc, CrossMab, (scFv)₂-Fc, scFv/scFv-Fc, Fab/(scFv)₂-Fc, Fab/Fab-scFv-Fc (= IgG-central scFv), Fab/Fab-crossFab-Fc, Fab/crossFab-Fc, and IgG-scFv, IgG-(scFv)₂.

13. The antibody of any one of embodiments 1 to 12 for use in the treatment or
10 detection of malignancies, wherein said antibody is administered to a subject in a first step and a HSG moiety is administered in a second step, wherein said HSG moiety binds to the antibody.

14. The antibody for use of embodiment 13, wherein said HSG moiety is
conjugated to or labeled with one or more diagnostic and/or therapeutic agents,
specifically said HSG moiety comprises one or more HSG haptens, one or more
15 diagnostic and/or therapeutic agents, and optionally a chelator.

15. The antibody of any one of embodiments 1 to 12, wherein said antibody is
bound to a HSG moiety conjugated to or labeled with one or more diagnostic and/or
therapeutic agents, specifically said HSG moiety comprises one or more HSG haptens,
one or more diagnostic and/or therapeutic agents, and optionally a chelator.

20 16. The antibody for use of embodiment 13 or 14, or the antibody of embodiment 15, wherein the therapeutic agent, is selected from a radionuclide, a chemotherapeutic agent, and a cytokine; and the diagnostic agent is a radionuclide.

17. The antibody of embodiment 16, wherein the chelator binds a radionuclide
and is specifically selected from DOTA, DTPA, deferoxamine B (DFO) and DFO*.

25 18. The antibody of embodiment 16 or 17, wherein the radionuclide is selected from the group consisting of ¹¹C, ¹³N, ¹⁵O, ¹⁸F, ³²P, ³³P, ⁴⁷Sc, ⁵⁹Fe, ⁶⁴Cu, ⁶⁷Cu, ⁶⁷Ga, ⁷⁵Se, ⁷⁷As, ⁸⁹Sr, ⁸⁹Zr, ⁹⁰Y, ^{99m}Tc, ⁹⁹Mo, ¹⁰³Pd, ¹⁰⁵Rh, ¹⁰⁹Pd, ¹¹¹Ag, ¹¹¹In, ¹²³I, ¹²⁴I, ¹²⁵I, ¹³¹I, ¹⁴⁰La, ¹⁴²Pr, ¹⁴³Pr, ¹⁴⁹Tb, ¹⁴⁹Pm, ¹⁵³Sm, ¹⁵⁹Gd, ¹⁶¹Tb, ¹⁶⁵Dy, ¹⁶⁶Dy, ¹⁶⁶Ho, ¹⁶⁹Yb, ¹⁶⁹Er, ¹⁷⁵Yb, ¹⁷⁷Lu, ¹⁸⁶Re, ¹⁸⁸Re, ¹⁸⁹Re, ¹⁹²Ir, ^{193m}Pt, ^{195m}Pt, ¹⁹⁴Ir, ¹⁹⁸Au, ¹⁹⁹Au, ²¹¹At, ²¹¹Pb, ²¹²Pb, ²¹²Bi, ²¹³Bi, ²¹¹At, ²²³Ra, ²²⁵Ac, and ²²⁷Th.
30

19. The antibody of any one of embodiments 1 to 12, for use in the preparation
of a medicament.

20. A pharmaceutical composition comprising the antibody of any one of
embodiments 1 to 12 together with a pharmaceutical excipient.

21. The pharmaceutical composition of embodiment 20, wherein said pharmaceutical composition is formulated for intravenous administration.

22. The pharmaceutical composition of embodiment 20 or 21 for use in the treatment of a patient suffering from cancer, specifically the treatment of tumors, solid
5 tumors, more specifically the treatment of colorectal cancer, ovarian cancer, breast cancer, prostate cancer, pancreas cancer, and lung cancer.

23. An isolated nucleic acid encoding the antibody of any one of embodiments 1 to 12.

24. An expression vector comprising the nucleic acid of embodiment 23.

10 25. A method for in vivo diagnosing cancer in a subject, wherein the antibody of any one of embodiments 1 to 12, or the antibody of any one of embodiments 15 to 18 is used for detecting tumor cells.

26. A method for in vitro diagnosing cancer, wherein the antibody of any one of embodiments 1 to 12, or the antibody of any one of embodiments 15 to 18 is used for
15 detecting tumor cells in a sample.

27. Method for treating cancer using the antibody of any one of embodiments 1 to 12, or the pharmaceutical composition of embodiment 20 or 21.

EXAMPLES

20 The examples described herein are illustrative of the present inventions. Many modifications and variations may be made to the techniques described and illustrated herein without departing from scope of the invention. Accordingly, it is understood that the examples are illustrative only and are not limiting the scope of the invention.

25 **Example 1:** Schematic drawings of exemplary anti-tumor antigen x anti-HSG bispecific mAbs, and amino acid sequences of anti-oxMIF x anti-HSG bispecific mAbs.

Figure 1 provides a schematic drawing of anti-Target X x anti-HSG bispecific mAbs having Fab-scFv-Fc, CrossMab (CH1-CL crossover), and IgG-central scFv formats. Left: Fab-scFv-Fc, central: CrossMab (CH1-CL crossover), right: IgG-central scFv.

30

Table I. Anti-HSG VH sequences & respective closest human germlines.

SEQ ID NO:	Description	Sequence
1	VH mo679*	EVILVESGGDLVKPGGSLKLSCAASGFTFSIYTM SWLRQTPE KRLEWWATL SGDGDDIYPDSVKGRFTISRDN AKNNLYLQM NSLRSADTALYYCARVRLGDWDFDVWGQGTTSVSS
2	VH hz679**	EVQLVESGGDLVKPGGSLKLSCAASGFTFSIYTM SWLRQTP GKGLEWWATL SGDGDDIYPDSVKGRFTISR DN AKNSLYLQ MNSLRAEDTALYYCARVRLGDWDFDVWGQGTTSVSS
3	IGHV3-21*07	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMN WVRQA PGKGLEWSSIS SSSGSTIYYADSVKGRFTISR DN AKNSLYLQ MNSLRAEDTAVYYCAR
4	hzVH1	EVQLLESGGGLVQPGGSLRLSCAASGFTFSIYTM SWVRQAP GKGLEWWSTL SGDGDDIYPDSVKGRFTISR DN AKNSLYLQ MNSLRAEDTAVYYCARVRLGDWDFDVWGQGTMTVSS
5	IGHV3-48*03	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYEMN WVRQA PGKGLEWWSYIS SSSGSTIYYADSVKGRFTISR DN AKNSLYLQ MNSLRAEDTAVYYCAR
6	hzVH2	EVQLVESGGDLVQPGGSLRLSCAASGFTFSIYTM SWVRQA PGKGLEWWATL SGDGDDIYPDSVKGRFTISR DN AKNNLYLQ QMNSLRSADTALYYCARVRLGDWDFDVWGQGTTVTVSS
7	IGHV3-7*01	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWM SWVRQ APGKGLEWWANIKQDGSEKYYVDSVKGRFTISRDN AKNSLYLQ LQMNSLRAEDTAVYYCAR
8	hzVH3	QVQLVESGGDLVKPGGSLRLSCAASGFTFSIYTM SWLRQTP EKRLEWWSTL SGDGDDIYPDSVKGRFTISR DN AKNSLYLQ MNSLRAEDTALYYCARVRLGDWDFDVWGQGT LVTVSS
9	IGHV3-11*01	QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYYMS WIRQA PGKGLEWWSYIS SSSGSTIYYADSVKGRFTISR DN AKNSLYLQ MNSLRAEDTAVYYCAR
10	hzVH4	EVQLVESGGGLVKPGGSLRLSCAASGFTFSIYTM SWLRQTP EKRLEWWSTL SGDGDDIYPDSVKGRFTISR DN AKNSLYLQ MNSLRAEDTAVYYCARVRLGDWDFDVWGQGTTSVSS
11	IGHV3-21*07	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMN WVRQA PGKGLEWSSIS SSSGSTIYYADSVKGRFTISR DN AKNSLYLQ MNSLRAEDTAVYYCAR
12	hzVH5	EVQLVESGGDLVKPGGSLKLSCAASGFTFSIYTM SWVRQAP EKRLEWWSTL SGDGDDIYPDSVKGRFTISR DN AKNSLYLQ MNSLRAEDTAVYYCARVRLGDWDFDVWGQGT TVTVSS
13	IGHV3-21*07	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMN WVRQA PGKGLEWSSIS SSSGSTIYYADSVKGRFTISR DN AKNSLYLQ MNSLRAEDTAVYYCAR

*US2009/0246131A1; **US2009/0240037A; CDRs are underlined and defined using combined IMGT/Kabat antibody numbering systems.

Table II. Anti-HSG VL sequences & respective closest human germlines.

SEQ ID NO:	Description	Sequence
14	VL mo679*	DIVMSQSPSSLAVSPGEKVTMTCKSSQSLFNSRTRKNYLGWY QKPGQSPKLLIYWASTRESGVDPDRFTGSGSGTDFTLTINSVQ SEDLAVYYCTQVYYLCTFGAGTKLELK
15	VL hz679**	DIVMTQSPSSLAVSPGERVTLTCKSSQSLFNSRTRKNYLGWYQ QKPGQSPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTINSLQA EDVAVYYCTQVYYLCTFGAGTKLELK
16	IGKV4-1*01	DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQ QKPGQPPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTISLQA EDVAVYYCQQYYSTP
17	hzVL1	DVVMQSPSSLAVSLGERVTINCKSSQSLFNSRTRKNYLGWYQ QKPGQSPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTISLQS EDVAVYYCTQVYYLCTFGQGTKLEIK
18	IGKV4-1*01	DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQ QKPGQPPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTISLQA EDVAVYYCQQYYSTP
19	hzVL2	DIVMTQSPSSLAVSLGERATITCKSSQSLFNSRTRKNYLGWYQ QKPGQPPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTINSLQS EDLAVYYCTQVYYLCTFGQGTKLEIK
20	IGKV4-1*01	DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQ QKPGQPPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTISLQA EDVAVYYCQQYYSTP
21	hzVL3	DIVMTQSPSLPVPVPGEKASISCKSSQSLFNSRTRKNYLGWYQ QKPGQSPKLLIYWASTRESGVDPDRFSGSGSGTDFTLKISRVEA EDVAVYYCTQVYYLCTFGAGTKLELK
22	IGKV2-28*01	DIVMTQSPSLPVPVPGEPAISCRSSQSLHNSGYNLDWYLQ KPGQSPQLLIYLGSNRASGVDPDRFSGSGSGTDFTLKISRVEAE DVGVIYCMQALQTP
23	hzVL4	DIQMTQSPSSLSASLGDRVTITCKSSQSLFNSRTRKNYLGWYQ QKPGQVPKLLIYWASTRESGVPSRFSGSGSGTDFTLTISLQS EDVAVYYCTQVYYLCTFGAGTKLELK
24	IGKV4-1*01	DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQ QKPGQPPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTISLQA EDVAVYYCQQYYSTP
25	hzVL5	DIVMTQSPDSLAVSPGERATITCKSSQSLFNSRTRKNYLGWYQ QKPGQSPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTINSLQA EDLAVYYCTQVYYLCTFGAGTKLELK
26	IGKV4-1*01	DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQ QKPGQPPKLLIYWASTRESGVDPDRFSGSGSGTDFTLTISLQA EDVAVYYCQQYYSTP
89	hzVL3.1	DIVMTQSPSLPVPVPGEKASISCKSSQSLFNSRTRKNYLGWYQ QKPGQSPKLLIYWASTRESGVDPDRFSGSGSGTDFTLKISRVEA EDVAVYYCTQVYYLCTFGAGTKLEIK

SEQ ID NO:	Description	Sequence
90	hzVL4.1	DIQMTQSPSSLSASLGDRVITITCKSSQSLFNSRTRKNYLGWYQ QKPGQVPKLLIYWASTRESGVPSRFSGSGSGTDFTLTISLQS EDVAVYYCTQVYYLCTFGAGTKLEIK
91	hzVL5.1	DIVMTQSPDSLAVSPGERATITCKSSQSLFNSRTRKNYLGWYQ QKPGQSPKLLIYWASTRESGVPDFSGSGSGTDFTLTINSLQA EDLAVYYCTQVYYLCTFGAGTKLEIK

*US2009/0246131A1; **US2009/0240037A; CDRs are underlined and defined using combined IMGT/Kabat antibody numbering systems.

Table III. anti-oxMIF mAb sequences, constant regions and linkers.

SEQ ID NO:	Description	Sequence
27	VL oxMIF*	DIQMTQSPSSLSASVGDRTITCRSSQRIMTYLNWYQQKPGKAPKLLI YVGSLSQSGVPSRFRGSGSETDFTLTISGLQPEDSATYYCQQSFFTP LTFGGGTKVEIK
28	VH oxMIF*	EVQLQESGGGLVQPGGSLRLSCAASGFTFSIYSMNWVRQAPGKGLE WVSSIGSSGGTTYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAV YYCAGSQYLYGMDVWGQGTTVTVSS
29	CH1 wt	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKV DKKV
30	CH1 cross	SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNT KVDKKVEPKSC
31	CH2-CH3 wt	APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMEALHNHYTQKSLSLSPG
32	CH2-CH3 KIH hole FcyR silent	APEAAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKG FYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRW QQGNVFSCSVMEALHNHYTQKSLSLSPG
33	Hinge-CH2- CH3 KIH hole FcyR/FcRn silent	DKTHTCPPCPAPEAAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVS HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPRDEL KNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFF LVSKLTVDKSRWQQGNVFSCSVMEALHNQYQKSLSLSPG
34	CH2-CH3 KIH hole FcyR/FcRn silent	APEAAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVCTLPSPRDELTKNQVSLSCAVKG

SEQ ID NO:	Description	Sequence
		FYPDSIAVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNQYTQKSLSLSPG
35	CH2-CH3 KIH knob FcyR silent	APEAAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPG
36	Hinge-CH2-CH3 KIH knob FcyR/FcRn silent	EPKSCDKTHTCPPCPAPEAAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNQYTQKSLSLSPG
37	CL kappa	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
38	CL kappa cross	ASVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQG LSSPVTKSFNRGEC
39	Linker 1 n = 3, 4 or 5	(GGS) _n
40	Truncated hinge n= 0 or 1	(D) _n KTHTCPPCP
41	Linker 3	EPKSCGGGGSGGGGS
42	Linker 4 n= 1, 2 or 3	(GGGGS) _n
43	Constant heavy chain (CH1+hinge+CH2+CH3) KIH knob FcyR silent	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPG
44	Linker+hinge+CH2-CH3 KIH hole FcyR silent	GGGGSDKTHTCPPCPAPEAAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPG
45	VL oxMIF (imalumab)	DIQMTQSPSSLSASVGDRVTITCRSSQRIMTYLNWYQQKPGKAPKLLIFVASHSQSGVPSRFRGSGSETDFTLTISGLQPEDSATYYCQQSFWTPLTFGGGTKEIK

SEQ ID NO:	Description	Sequence
46	VH oxMIF (imalumab)	EVQLLESGGGLVQPGGSLRLSCAASGFTFSIYSMNWVRQAPGKGLE WVSSIGSSGGTTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAV YYCAGSQWLYGMDVWGQGTTVTVSS

*WO/2022/069712A1; FcγR silent: mutations in mAb Fc region abolishing binding to FcγR and complement (***bold italicized***); FcRn silent: mutations in mAb Fc region reducing or abolishing binding to FcRn. (**bold underlined**); KIH (knob-into- hole): knob and hole mutations (**bold**)

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Table IV. anti-oxMIF x anti-HSG Fab-scFv-Fc humanized variants.

	VL1	VL2	VL3	VL4	VL5	VL hz679
VH1	C0176 (SEQ ID NOs: 27+37 28+43+ 4+39+17+44)	C0177 (SEQ ID NOs: 27+37+ 28+43+ 4+39+19+44)	C0178 (SEQ ID NOs: 27+37+ 28+43+ 4+39+21+44)	C0179 (SEQ ID NOs: 27+37+ 28+43+ 4+39+23+44)	C0180 (SEQ ID NOs: 27+37+ 28+43+ 4+39+25+44)	
	C0181 (SEQ ID NOs: 27+37+ 28+43+ 6+39+17+44)	C0182 (SEQ ID NOs: 27+37+ 28+43+ 6+39+19+44)	C0183 (SEQ ID NOs: 27+37+ 28+43+ 6+39+21+44)	C0184 (SEQ ID NOs: 27+37+ 28+43+ 6+39+23+44)	C0185 (SEQ ID NOs: 27+37+ 28+43+ 6+39+25+44)	
VH2						
VH3	C0186 (SEQ ID NOs: 27+37+ 28+43+ 8+39+17+44)	C0187 (SEQ ID NOs: 27+37+ 28+43+ 8+39+19+44)	C0188 (SEQ ID NOs: 27+37+ 28+43+ 8+39+21+44)	C0189 (SEQ ID NOs: 27+37+ 28+43+ 8+39+23+44)	C0190 (SEQ ID NOs: 27+37+ 28+43+ 8+39+25+44)	
	C0191 (SEQ ID NOs: 27+37+ 28+43+ 10+39+17+44)	C0192 (SEQ ID NOs: 27+37+ 28+43+ 10+39+19+44)	C0193 (SEQ ID NOs: 27+37+ 28+43+ 10+39+21+44)	C0194 (SEQ ID NOs: 27+37+ 28+43+ 10+39+23+44)	C0195 (SEQ ID NOs: 27+37+ 28+43+ 10+39+25+44)	
VH4						
VH5	C0196 (SEQ ID NOs: 27+37+ 28+43+ 12+39+17+44)	C0197 (SEQ ID NOs: 27+37+ 28+43+ 12+39+19+44)	C0198 (SEQ ID NOs: 27+37+ 28+43+ 12+39+21+44)	C0199 (SEQ ID NOs: 27+37+ 28+43+ 12+39+23+44)	C0200 (SEQ ID NOs: 27+37+ 28+43+ 12+39+25+44)	
	C0132 (SEQ ID NOs: 27+37+ 28+43+ 2+39+15+44)					

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Table V. anti- oxMIF x anti-HSG Crossmab humanized variants.

	VL1	VL2	VL3	VL4	VL hz679
VH1	C0238 (SEQ ID NOs: 27+37+ 28+29+36+ 17+30+ 4+38+33)	C0250 (SEQ ID NOs: 27+37+ 28+29+36+ 19+30+ 4+38+33)	C0251 (SEQ ID NOs: 27 +37+ 28+29+36+ 21+30+ 4+38+33)	C0252 (SEQ ID NOs: 27+37+ 28+29+36+ 23+30+ 4+38+33)	
	C0239 (SEQ ID NOs: 27+37+ 28+29+36+ 17+30+ 6+38+33)	C0240 (SEQ ID NOs: 27+37+ 28+29+36+ 19+30 + 6+38+33)	C0248 (SEQ ID NOs: 27+37+ 28+29+36+ 21+30+ 6+38+33)	C0249 (SEQ ID NOs: 27+37+ 28+29+36+ 23+30+ 6+38+33)	
VH2					
VH3	C0241 (SEQ ID NOs: 27+37+ 28+29+36+ 17+30+ 8+38+33)	C0245 (SEQ ID NOs: 27+37+ 28+29+36+ 19+30+ 8+38+33)	C0246 (SEQ ID NOs: 27+37+ 28+29+36+ 21+30+ 8+38+33)	C0247 (SEQ ID NOs: 27+37+ 28+29+36+ 23+30+ 8+38+33)	
VH hz679					C0255 (SEQ ID NOs: 27+37+ 28+29+36+ 15+30+ 2+38+33)

Table VI. anti- oxMIF x anti-HSG Crossmab humanized variants comprising the humanized VL3.1-VL5.1 sequences.

	VL3.1	VL4.1	VL5.1
VH1	C0251.1 (SEQ ID NOs: 27 +37+ 28+29+36+ 89+30+ 4+38+33)	C0252.1 (SEQ ID NOs: 27+37+ 28+29+36+ 90+30+ 4+38+33)	(SEQ ID NOs: 27+37+ 28+29+36+ 91+30+ 4+38+33)
	C0248.1 (SEQ ID NOs: 27+37+ 28+29+36+ 89+30+ 6+38+33)	C0249.1 (SEQ ID NOs: 27+37+ 28+29+36+ 90+30+ 6+38+33)	(SEQ ID NOs: 27+37+ 28+29+36+ 91+30+ 6+38+33)
VH2			
VH3	C0246.1 (SEQ ID NOs: 27+37+ 28+29+36+ 89+30+ 8+38+33)	C0247.1 (SEQ ID NOs: 27+37+ 28+29+36+ 90+30+ 8+38+33)	(SEQ ID NOs: 27+37+ 28+29+36+ 91+30+ 8+38+33)

C0181**Polypeptide 1 (LC oxMIF) SEQ ID NO: 47**

DIQMTQSPSSLSASVGDRVITICRSSQRIMTYLNWYQQKPGKAPKLLIYVGSHSQSG
VPSRFRGSGSETDFTLTISGLQPEDSATYYCQQSFFTPLTFGGGTKVEIKRTVAAPSV
5 FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Polypeptide 2 (HC oxMIF) SEQ ID NO: 48

EVQLQESGGGLVQPGGSLRLSCAASGFTFSIYSMNWVRQAPGKGLEWVSSIGSSGG
TTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAGSQYLYGMDVWGQGT
10 TVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKHTC
PPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
GQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTP
15 VLDSGGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

Polypeptide 3 (HSG HC) SEQ ID NO: 49

EVQLVESGGDLVQPGGSLRLSCAASGFTFSIYTMSWVRQAPGKGLEWVATLSGDGD
DIYYPDSVKGRFTISRDN AKNNLYLQMNSLRSADTALYYCARVRLGDWDFDVGQG
TTTVSSGGSGGSGGSGGSGGSDVMTQSPSSLAVSLGERVTINCKSSQSLFNSRT
20 RKNYLGWYQQKPGQSPKLLIYWASTRESGVPDRFSGSGSGTDFTLTISLQSEDAV
YYCTQVYYLCTFGQGTKLEIKGGGSGDKHTCPCPAPEAAGGPSVFLFPPKPKDTL
MISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVS
LSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGGSFFLVSKLTVDKSRWQQG
25 NVFSCFSVMHEALHNHYTQKSLSLSPG

C0182**Polypeptide 1 (LC oxMIF) SEQ ID NO: 47****Polypeptide 2 (HC oxMIF) SEQ ID NO: 48****30 Polypeptide 3 (HC HSG) SEQ ID NO: 50**

EVQLVESGGDLVQPGGSLRLSCAASGFTFSIYTMSWVRQAPGKGLEWVATLSGDGD
DIYYPDSVKGRFTISRDN AKNNLYLQMNSLRSADTALYYCARVRLGDWDFDVGQG
TTTVSSGGSGGSGGSGGSGGSDIVMTQSPSSLAVSLGERATITCKSSQSLFNSRTR
KNYLGWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTINSLQSEDLAVY

YCTQVYYLCTFGQGKLEIKGGGGSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM
ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSL
CAVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNV
5 FSCSVMHEALHNHYTQKSLSLSPG

C0186**Polypeptide 1 (LC oxMIF) SEQ ID NO: 47****Polypeptide 2 (HC oxMIF) SEQ ID NO: 48****10 Polypeptide 3 (HC HSG) SEQ ID NO: 51**

QVQLVESGGDLVKPGGSLRLSCAASGFTFSIYMSWLRQTPEKRLEWVSTLSGDGD
DIYYPDSVKGRFTISRDNANKNSLYLQMNSLRAEDTALYYCARVRLGDWDFDVWGQG
TLVTVSSGGSGGSGGSGGSGGSDVVMQSPSSLAVSLGERVTINCKSSQSLFNSRT
RKNYLGWYQQKPGQSPKLLIYWASTRESGVPDRFSGSGSGTDFTLTISLQSEDAV
15 YYCTQVYYLCTFGQGKLEIKGGGGSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVS
LSCAVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQG
NVFSCSVMHEALHNHYTQKSLSLSPG

20

C0192**Polypeptide 1 (LC oxMIF) SEQ ID NO: 47****Polypeptide 2 (HC oxMIF) SEQ ID NO: 48****Polypeptide 3 (HC HSG) SEQ ID NO: 52**

25 EVQLVESGGGLVKPGGSLRLSCAASGFTFSIYMSWLRQTPEKRLEWVSTLSGDGD
DIYYPDSVKGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCARVRLGDWDFDVWGQG
TTVSVSSGGSGGSGGSGGSGGSDIVMTQSPSSLAVSLGERATITCKSSQSLFNSRTR
KNYLGWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTINSLQSEDLAVY
YCTQVYYLCTFGQGKLEIKGGGGSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLM
30 ISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSL
CAVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSRWQQGNV
FSCSVMHEALHNHYTQKSLSLSPG

oxMIF epitope

EPCALCS SEQ ID NO: 53.

C02395 **Polypeptide 1 (LC oxMIF) SEQ ID NO: 47****Polypeptide 2 (HC oxMIF) SEQ ID NO: 54**

EVQLQESGGGLVQPGGSLRLSCAASGFTFSIYSMNWVRQAPGKGLEWSSIGSSGG
TTYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAGSQYLYGMDVWGQGT
TVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
10 TFAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC
PPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLAQDWLNGKEYKCKVSNKALPAPIEKTISKAKG
QPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTPPV
LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNQYTQKSLSLSPG

15 **Polypeptide 3 (HC HSG) SEQ ID NO: 55**

EVQLVESGGDLVQPGGSLRLSCAASGFTFSIYTMSWVRQAPGKGLEWWATLSGDGD
DIYYPDSVKGRFTISRDN AKNNLYLQMNSLRSADTALYYCARVRLGDWDFDVGQG
TTTVSSASVAAPSVFIFPPSDEQLKSGTASVCLLNNFYPPREAKVQWKVDNALQSG
NSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
20 DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNW
YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQDWLNGKEYKCKVSNKALPAPIEK
TISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNY
KTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNQYTQKSLSLSPG

Polypeptide 4 (LC HSG) SEQ ID NO: 56

25 DVVMTQSPSSLAVSLGERVTINCKSSQSLFNSRTRKNYLGWYQQKPGQSPKLLIYWA
STRESGVPDRFSGSGSGTDFTLTISSLQSEDAVYYCTQVYYLCTFGGQGTKLEIKSSA
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ
SSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

C024030 **Polypeptide 1 (LC oxMIF) SEQ ID NO: 47****Polypeptide 2 (HC oxMIF) SEQ ID NO: 54****Polypeptide 3 (HC HSG) SEQ ID NO: 55****Polypeptide 4 (LC HSG) SEQ ID NO: 57**

DIVMTQSPSSLAVSLGERATITCKSSQSLFNSRTRKNYLGWYQQKPGQPPKLLIYWA
 STRESGVPDRFSGSGSGTDFTLTINSLSQSEDLAVYYCTQVYYLCTFGQGKLEIKSSA
 STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ
 SSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

5

Figure 1 shows exemplary Fab-scFv-Fc, CrossMab and IgG-central scFv formats of the bispecific anti-tumor Target X x anti-HSG mAbs. **Table I & II** show anti-HSG VH (**Table I**) and anti-HSG VL (**Table II**) sequences and their respective closest human germlines. **Table III** shows anti-oxMIF mAb sequences, mAb constant regions and
 10 linkers. **Table IV** shows anti- oxMIF x anti-HSG humanized variants in Fab-scFv-Fc format. **Table V** shows anti- oxMIF x anti-HSG humanized variants in Crossmab format.

Example 2: *In silico* immunogenicity (humanness score) of the VH & VL variants 1-5 of the newly humanized anti-HSG sequences *versus* hz679.

15 To generate novel humanized VH and VL sequences the CDRs of the murine anti-HSG antibody mo679 (from US 20090246131 A1) were identified using a combination of IMGT and Kabat numbering systems which enables optimal retention of CDR-loop conformation. Databases of human IgG1/Igk sequences were searched for comparison to the murine VH and VL regions of mo679 using IgBLAST search
 20 algorithms (NCBI), and candidate human VH & VL domains were selected from the top 200 BLAST results. These were reduced to several candidates based on a combination of framework homology, maintaining key framework residues and canonical loop structure. Murine CDRs of mo679 were grafted into defined human frameworks as “acceptor” frameworks, resulting in 5 newly humanized VH (VH1-VH5, see **Table I**) and
 25 8 newly humanized VL variants (VL1, VL2, VL3, VL4, VL5, VL3.1, VL4.1, VL5.1, see **Table II**).

Determination of the antibody humanness: The variable (V) regions of the newly humanized variants (VH1-VH5 and VL1-VL5, VL3.1, VL4.1, VL5.1) and the previously humanized variable (V) regions of anti-HSG antibody hz679 (from US 2009/0240037
 30 A1) were analyzed to determine: 1) their percentage of identity to the closest human germline and 2) their humanness according to WHO’s definition of humanized antibodies by Immunogenetics Information System® (IMGT®) DomainGapAlign tool, <https://www.imgt.org/3Dstructure-DB/cgi/DomainGapAlign.cgi>).

Results & Conclusion: As evident from the **Table VII** and **Table VIII**, all 5 newly humanized VH-variants (VH1-5) and 4 of the newly humanized VL variants (VL1, VL2, VL5, and VL5.1) show a higher degree of identity (%) to their respective closest human germlines when compared to the previously humanized anti-HSG antibody hz679 and its closest human germline (**Table VII**). Furthermore, the 2 newly humanized VH-variants VH1 and VH2, as well as the 4 newly humanized VL-variants VL1, VL2, VL5, and VL5.1 are defined as fully human, according to WHO's definition of humanized antibodies (**Table VIII**). The newly humanized VH-variants VH3 and VH4 are equally close to human and to mouse, the VH5 is equally close to human, mouse and monkey, whereas the newly humanized VL-variants VL3, VL3.1, VL4, and VL4.1 are closest to monkey (**Table VIII**). In contrast, the previously humanized anti-HSG antibody hz679 is still closer to mouse according to the WHO's definition of humanized antibodies (**Table VIII**). It is well known in the art, that the closer the sequence of an antibody to its closest human germline is, the less immunogenic the antibody will be once administered into humans (Gao SH et al., 2013). Therefore, the increased humanness score for the newly humanized anti-HSG VH- and VL-variants reduces the immunogenicity potential of antibodies comprising the newly humanized variable domain sequences.

Table VII. % Identity to the closest human germlines.

Variant	Variable (V) region	Joining (J) region	Closest human germline(s)
VH hz679*	83.7	85.7	IGHV3-21*07, IGHV3-11*01, IGHV3-11*04, IGHV3-7*01, IGHV3-7*02
VH1	88.8	100	IGHV3-48*03
VH2	84.7	92.9	IGHV3-7*01, IGHV3-7*02, IGHV3-7*03, IGHV3-7*04, IGHV3-7*05
VH3	84.7	92.9	IGHV3-11*01, IGHV3-11*04
VH4	85.7	85.7	IGHV3-21*07, IGHV3-11*01, IGHV3-11*04
VH5	85.7	92.9	IGHV3-21*07
VH mo679**	77.6	85.7	IGHV3-21*07, IGHV3-11*01, IGHV3-11*04, IGHV3-7*01, IGHV3-7*02
VL hz Ab 679*	83.7	83.3	IGKV4-1*01, *02, *03
VL1	85.7	100	IGKV4-1*01, *02, *03
VL2	85.7	100	IGKV4-1*01, *02, *03
VL3	82.3	83.3	IGKV2-28*01, IGKV2D-28*01
VL3.1	82.3	91.7	IGKV2-28*01, IGKV2D-28*01
VL4	80.6	83.3	IGKV4-1*01, *02, *03
VL4.1	80.6	91.7	IGKV4-1*01, *02, *03
VL5	85.7	83.3	IGKV4-1*01, *02, *03
VL5.1	85.7	91.7	IGKV4-1*01, *02, *03
VL mo679**	77.6	83.3	IGKV4-1*01, *02, *03

*(US2009/0240037 A1), **(US2009/0246131 A1)

Table VIII. Humanness of anti-HSG VH and VL variant sequences according to the WHO's definition of humanized antibodies.

Variant	Closest species
VH hz679*	mouse
VH1	human
VH2	human
VH3	human or mouse
VH4	human or mouse
VH5	human or mouse or monkey
VH mo679**	mouse
VL hz679*	mouse
VL1	human or monkey
VL2	human
VL3	monkey
VL3.1	monkey
VL4	monkey
VL4.1	monkey
VL5	human
VL5.1	human
VL mo679**	mouse

*(US2009/0240037 A1), **(US2009/0246131 A1)

5 **Example 3:** Production and manufacturing properties of anti-oxMIF x anti-HSG bispecific antibodies.

Materials and Methods: For generation of novel anti-oxMIF x anti-HSG bispecific Fab-scFv-Fc mAbs, the newly humanized VH and VL sequences were linked to a scFv (VH-(GGG)₅-VL) (SEQ ID NO: 88) and were fused to a FcγR silenced human IgG1 “hole” Fc by a linker including a truncated hinge region (GGGGSDKTHTCPPCP, SEQ ID NO: 58) resulting in the anti-HSG scFv-Fc “hole” heavy chain. The VH and VL of the previously humanized anti-HSG antibody hz679 were used as reference. The plasmids containing synthetic anti-HSG scFv-Fc heavy chains (HCs) were co-transfected with plasmids encoding the respective anti-oxMIF antibody chains (LC and HC, FcγR silenced human IgG1 “knob” constant domain) in the ratio of 3:2:1 (anti-oxMIF LC: anti-oxMIF HC: anti-HSG scFv-Fc) into ExpiCHO-S cells. This resulted in anti-oxMIF x anti-HSG bispecific antibodies C0176-C0200 (comprising newly humanized anti-HSG sequences) and C0132 (comprising previously humanized anti-HSG sequences of hz679 (**Table IV**) upon the transient expression using the MAX Titer protocol (Thermo
20 Fisher Scientific).

For generation of anti-oxMIF x anti-HSG bispecific CrossMabs, the newly humanized anti-HSG VH sequences (VH1-VH3) were cloned in-frame with the constant region of FcγR/FcRn silent human IgG1 “hole” HC (CL_{kappa cross}-CH2-CH3) resulting in the anti-HSG HC, and the newly humanized anti-HSG VL sequences (VL1-VL4) were
5 cloned in-frame with the CH1 region of human IgG1 (CH1_{cross}), resulting in anti-HSG light chain. The VH and VL of the previously humanized anti-HSG antibody hz679 were used as reference. The plasmids containing synthetic anti-HSG heavy chains (HCs)) and light chains (LCs) were co-transfected with plasmids encoding the respective anti-oxMIF antibody chains (LC and HC, FcγR/FcRn silenced human IgG1 “knob” constant
10 domain) in the ratio of 2:1:1:2 (anti-oxMIF LC: anti-oxMIF HC: anti-HSG HC: anti-HSG LC). This resulted in anti-oxMIF x anti-HSG Crossmabs C0238-C0252 (comprising newly humanized anti-HSG sequences) and C0255 (comprising previously humanized anti-HSG sequences from hz679 (**Table V**).

Heterodimerization of the 2 different heavy chains was achieved by using knob-
15 into-hole (KIH) technology (anti-oxMIF HC “knob”: T366W and S354C mutation for disulfide bond stabilization; anti-HSG HC „hole“: T366S/L368A/Y407V and Y349C mutation for disulfide bond stabilization). In addition, all heavy chains carry L234A/L235A („LALA“) mutations to minimize the binding to Fcγ receptor-bearing immune cells and complement. Bispecific mAbs in the CrossMab format additionally comprise mutations
20 H310A/H435Q to modulate binding to human FcRn in order to optimize mAb half-life for pre-targeting in humans. Positions of mutations are numbered according to the EU numbering system.

ExpiCHO-S cultures were harvested 12 days post-transfection, and mAbs were purified from cell culture supernatants by protein A affinity chromatography (MabSelect
25 Prism A HiTrap column, Cytiva). MAbs were further purified by preparative CEX using Poros XS columns and were formulated in 10 mM sodium acetate/acetic acid, 200 mM NaCl, pH 5.8.

Mab expression titers were calculated as mg of protein A-purified mAb per liter of harvested cell culture supernatant (mg/L). The relative abundance of correctly
30 assembled bispecific mAbs (monomeric purity) was assessed by analytical size-exclusion chromatography (SEC) using an ENrich 650 column (Bio-Rad) and by analytical cation exchange chromatography (CEX) using Poros XS columns (Cytiva) of samples after protein A purification.

MAb purity and severity of anti-HSG heavy chain (scFv-Fc) cleavage was assessed by reducing NuPAGE™ 4-12% SDS-PAGE (Thermo Fisher Scientific, 3 µg/lane) and subsequent InstantBlue® Coomassie staining (Abcam). Band intensities (BI) were quantified by ImageStudioLite Version 5.2 software (LI-COR) and cleavage was calculated by following formula: (BI anti-HSG HC / BI anti-oxMIF LC)*100.

Results and Conclusion: Across the 25 Fab-scFv-Fc mAbs simultaneously expressed in 25 ml scale, mAbs C0176, C0181, C0182, C0186 and C0192, in which the newly humanized VL1 and VL2 variants of anti-HSG were paired with one of the newly humanized VH1-VH4 variants, revealed the best manufacturing characteristics as defined from a combination of their mAb expression titers, monomeric purity and severity of cleavage of the anti-HSG scFv-Fc heavy chain (candidates with arrows facing upwards - ↗ / ↑ - in **Table IX**). Mass spectrometry revealed that cleavage of anti-HSG scFv-Fc heavy chain occurred between the amino acid residues E105 and L106 (Kabat numbering) in the "KLELK" motif (SEQ ID NO: 59) of the FRW4 of the anti-HSG VL (see **Example 4**). Unexpectedly, antibodies containing VL sequences with variant variable domains VL3-5 (C0178-0180, C0183-0185, C0188-0190, C0193-0195, C0198-0200) sharing the same FR4 region (FGAGTKLELK, SEQ ID NO: 60) with the previously humanized anti-HSG mAb hz679 (C0132), showed significantly enhanced cleavage of the anti-HSG scFv HC (exemplified by C0178-C0180, **Figure 2**).

Thus, the top 5 ranked mAbs C0176, C0181, C0182, C0186 and C0192 were simultaneously re-expressed with the reference mAb C0132 having the sequence from previously humanized anti-HSG mAb hz679 in a larger scale (200 ml) to allow direct comparison of manufacturing properties. As evidenced by **Table X**, both expression titers and monomeric purity of the newly humanized bispecific mAbs C0176, C0181, C0182, C0186 and C0192 were significantly improved compared to C0132 having previously humanized anti-HSG sequences of hz679. In summary, bispecific Fab-scFv-Fc antibodies comprising the newly humanized anti-HSG variable regions **VL1-2** combined with **VH1-4** showed advantageous properties compared to the previously humanized anti-HSG mAb hz679.

The anti-oxMIF x anti-HSG bispecific CrossMabs C0238-C0252 were simultaneously expressed with a reference CrossMab C0255 having previously humanized anti-HSG sequence of hz679, in 100 ml-scale to allow head-to-head comparison. Crossmabs were purified as described earlier. As evident from the **Table XI**, the CrossMabs (C0238, C0239, C0240, C0241, C0245, C0250) in which the

newly humanized VH-variants (VH1-3) were paired with the newly humanized VL-variants (VL1-2), demonstrate improved manufacturing characteristics over the reference C0255. This improvement is evidenced by a combination of improved expression titers and improved monomeric purity by SEC and CEX (candidates with arrows facing upwards - ↗ / ↑ - in **Table XI**). In summary, again, bispecific Crossmabs comprising the newly humanized anti-HSG variable regions **VL1-2** combined with **VH1-3** showed advantageous properties compared to the reference C0255 comprising the previously humanized anti-HSG mAb hz679.

Table IX . Manufacturing characteristics newly humanized anti-oxMIF x anti-HSG bispecific Fab-scFv-Fc mAbs C0176-C0200.

Variant			Titer ¹ (mg/L)	Purity SEC ²	Purity CEX (% monomer)	Cleavage ³
C0176	VH1	VL1	↗ 156	↗ 194	↗ 76	↗ 11
C0177	VH1	VL2	↗ 136	↗ 199	↗ 75	↗ 13
C0178	VH1	VL3	↓ 114	↓ 115	↓ 38	↓ 56
C0179	VH1	VL4	↓ 114	↓ 109	↓ 36	↓ 55
C0180	VH1	VL5	↓ 70	↓ 115	↗ 60	↓ 33
C0181	VH2	VL1	↗ 142	↗ 231	↗ 77	↗ 11
C0182	VH2	VL2	↗ 132	↗ 239	↗ 76	↗ 11
C0183	VH2	VL3	↓ 113	↓ 93	↗ 56	↓ 42
C0184	VH2	VL4	↗ 141	↓ 92	↗ 57	↓ 37
C0185	VH2	VL5	↗ 161	↓ 145	↗ 70	↘ 18
C0186	VH3	VL1	↗ 164	↗ 208	↗ 80	↗ 7
C0187	VH3	VL2	↗ 139	↗ 185	↗ 79	↗ 7
C0188	VH3	VL3	↗ 122	↓ 113	↗ 62	x
C0189	VH3	VL4	↗ 128	↓ 103	↗ 55	↓ 39
C0190	VH3	VL5	↗ 126	↓ 135	↗ 76	x
C0191	VH4	VL1	↗ 178	↗ 231	↗ 77	↗ 6
C0192	VH4	VL2	↗ 180	↗ 214	↗ 75	↗ 12
C0193	VH4	VL3	↗ 148	↓ 114	↗ 54	↓ 24
C0194	VH4	VL4	↗ 140	↓ 120	↗ 51	↓ 26
C0195	VH4	VL5	↗ 137	↗ 158	↗ 76	↘ 17
C0196	VH5	VL1	↗ 126	↗ 159	↗ 70	↘ 20
C0197	VH5	VL2	↗ 135	↗ 163	↗ 69	↘ 22
C0198	VH5	VL3	↓ 58	↓ 136	↓ 48	↓ 56
C0199	VH5	VL4	↓ 76	↗ 182	↓ 17	↓ 69
C0200	VH5	VL5	↓ 81	↓ 74	↗ 55	↓ 36

¹ mg of Protein A purified mAb per L of cell culture supernatant; ² Calculated abundances of correctly assembled mAb monomer from integrated peak area of SEC chromatogram

(%) divided by peak width (mL); ³ Band intensities (BI) of cleaved anti-HSG heavy chain normalized by BI of anti-oxMIF light chain (BI anti-HSG HC / BI anti-oxMIF LC x 100). To determine the best candidates, expression titers and purity values were normalized that all values range from 0-100% (highest/best value = 100%, lowest value = 0%): ↑ ≥75%, ↗ ≥ 50 to 75%, ↓ < 50%. For cleavage: ↑ <12.5%, ↘ ≥ 12.5 to < 25% %, ↓ ≥25%. Figure 2 shows the assessment of mAb C0132 and C0176-C0180 purity and severity of cleavage of anti-HSG scFv-Fc heavy chain by SDS-PAGE and Coomassie staining. A total of 3 µg of Protein A purified mAbs were resolved by NuPAGE™ 4-12% SDS-PAGE under reducing conditions ("red."). Spectra Multicolor Broad Range Protein Ladder was used as standard. Arrows show the anti-oxMIF LC and the cleaved anti-HSG heavy chain.

Table X. Manufacturing characteristics of selected newly humanized Fab-scFv-Fc mAbs C0176, C0181, C0182, C0186, C0192 *versus* the reference mAb C0132 comprising previously humanized anti-HSG sequences of hz679.

Variant			Titer ¹ (mg/L)	Purity SEC (% monomer)
C0176	VH1	VL1	↗ 256	↗ 89
C0181	VH2	VL1	↗ 251	↑ 91
C0182	VH2	VL2	↑ 319	↑ 91
C0186	VH3	VL1	↑ 317	↗ 82
C0192	VH4	VL2	↑ 351	↗ 87
C0132	VH hz679	VL hz679	→ 235	→ 80

¹ mg of Protein A purified mAb per L of cell culture supernatant; ↑ ≥300, ↗ ≥ 250 to < 300, → < 250 mg/L; ² Calculated abundances of correctly assembled mAb monomer from integrated peak area of SEC chromatogram (%); ↑ ≥90, ↗ ≥ 81 to < 90, → ≤ 80 %.

Table XI. Manufacturing characteristics of newly humanized anti-oxMIF x anti-HSG bispecific CrossMabs C0238-C0252 *versus* the reference anti-oxMIF x anti-HSG CrossMab C0255 having previously humanized anti-HSG sequences of hz679.

Variant			Titer ¹ (mg/L)	Purity SEC ² (% monomer)	Purity CEX ² (% monomer)
C0238	VH1	VL1	↗ 133	↗ 82	↗ 53
C0250	VH1	VL2	↑ 159	↑ 92	↗ 51
C0251	VH1	VL3	↑ 170	↗ 84	↓ 38
C0252	VH1	VL4	↓ 116	→ 77	↗ 56
C0239	VH2	VL1	↑ 176	↗ 86	↗ 57
C0240	VH2	VL2	↑ 154	↗ 86	↑ 66
C0248	VH2	VL3	→ 128	↗ 86	↗ 59
C0249	VH2	VL4	↓ 111	↓ 75	↑ 60
C0241	VH3	VL1	↑ 150	↗ 88	↗ 51
C0245	VH3	VL2	→ 123	↗ 88	↗ 50
C0246	VH3	VL3	↓ 51	↗ 87	↑ 68
C0247	VH3	VL4	↑ 155	↗ 86	↓ 30
C0255	VH hz679	VL hz679	→ 123	→ 77	x

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¹ mg of Protein A purified mAb per L of cell culture supernatant; ² Calculated abundances of correctly assembled mAb monomer from integrated peak area of SEC chromatogram (%) or CEX chromatogram (%). To determine the best candidates, candidates were ranked as follows: Titer, ↑ ≥150, ↗ ≥ 130 < 150, → ≥ 123 <130, ↓ < 123 mg/L; purity by
 10 SEC: ↑ ≥90, ↗ ≥ 80 < 90, → ≥ 77 < 80, ↓ < 77 %; purity by CEX: ↑ ≥60, ↗ ≥ 50 < 60, → ≥ 40 < 50, ↓ < 40 %

Example 4: Assessment of the anti-HSG scFv-Fc heavy chain cleavage in anti-oxMIF x anti-HSG bispecific mAb C0132 by mass-spectrometry.

15 As described in **Example 3**, cleavage of anti-HSG scFv-Fc heavy chain was observed for all bispecific Fab-scFv-Fc anti-oxMIF x anti-HSG mAbs with varying severity. Unexpectedly, antibodies containing VL sequences with variant variable domains VL3-5 (C0178-0180, C0183-0185, C0188-0190, C0193-195, C0198-0200) sharing the same FR4 region (FGAGTKLELK; SEQ ID NO: 60) with the previously
 20 humanized anti-HSG mAb hz679 (C0132), showed significantly enhanced cleavage of the anti-HSG scFv HC (see **Table IX** and **Figure 2**) and this was much less pronounced for the variants comprising the newly humanized variants VL1 and VL2 compared to C0132. In the present example, a protein A and CEX purified C0132 fraction containing

high quantities of the truncated anti-HSG HC was subjected to mass-spectrometric analysis, to characterize the cleavage site in more detail.

Material and Methods: C0132 was diluted in 0.1% formic acid (FA) and separated on an Ultra Performance Liquid Chromatography (UPLC) system (Waters ACQUITY Premier) using a reversed phase column (MAbPac RP 4 μ m 2.1 x 50 mm, Thermo Scientific). Eluents were 0.1% FA in water and 0.1% FA in acetonitrile. The mass spectrometric analysis was performed with a Compact Quadrupol Time of Flight (QTOF) mass spectrometer (Bruker Daltonik). For data analysis, the recorded Liquid Chromatography-ElectroSpray Ionization-Mass Spectrometry (LC-ESI-MS) spectra were summed, deconvoluted and smoothed using the software DataAnalysis (Bruker Daltonik).

Results and Conclusion: **Figure 3** shows the intact mass spectrum of C0132 mAb. It reveals two main peaks at 126882.3 and 100701.9 Da, which corresponds to the intact antibody and the truncated HC respectively. The molecular mass of 100701.9 Da can only be obtained from anti-HSG scFv-Fc HC with a truncation of amino acids 1-244, meaning that cleavage occurs between the amino acid residues E105 and L106 (Kabat numbering) in the "KLEELK" motif of the FRW4 of the anti-HSG VL, present in newly humanized variants containing VL3-5 and previously humanized hz679 mAb. In contrast, Fab-scFv-Fc mAbs C0176, C0181, C0182, C0186 and C0192 which show only minor cleavage of the anti-HSG scFv-Fc all comprise the newly humanized VL-variants VL1 or VL2 carrying "KLEIK"-motif (SEQ ID NO: 61) in their FRW4.

Figure 3 shows a deconvoluted mass spectrum of C0132 mAb. The 2 main peaks correspond to the intact antibody (126882.3 Da) and to the antibody with the truncated anti-HSG scFv-Fc heavy chain (100701.9 Da).

Example 5: Stability of newly humanized anti-oxMIF x anti-HSG bispecific Fab-scFv-Fc mAbs C0176, C0181, C0182, C0186, and C0192 compared to a reference antibody having the VH&VL sequences of the previously humanized anti-HSG Ab hz679, upon different storage conditions.

Material and Methods: Stability of antibodies was assessment by SEC at day 0 and after storage for 83 days at -80°C (**Figure 4A**) or 4°C (**Figure 4B**). For SEC, samples were diluted to 1 mg/ml in the running buffer (0.1 M Phosphate Buffer, 0.2 M Arginine, pH 6.8), and 100 μ L sample was applied to an Enrich 650 (Bio-Rad) gel-filtration column at a flow rate of 1.25 mL/min. Protein peaks were monitored using

absorbance at 280 nm and spectra were analyzed using the ChromLab software package (Bio-Rad). Absorbance at 280 nm was used to integrate the peak area, and the relative areas of the different peaks were used to determine abundance of aggregates, monomers and fragments. To allow direct comparison of the samples, values for %
5 monomer were normalized to day 0 (= 100%) for each antibody.

Results and Conclusion: As illustrated in the **Figure 4**, the bispecific antibodies comprising newly humanized anti-HSG sequences demonstrated either improved (-80°C (**Figure 4A**): C0176, C0181, C0182, C0186, C0192; 4°C (**Figure 4B**): C0186 and C0192) or equivalent stability (4°C (**Figure 4B**): C0181 and C0182), evidenced from
10 either higher or similar monomeric content compared to C0132 (comprising the previously humanized anti-HSG sequence). In summary, again, novel bispecific antibodies containing the newly humanized VL-variants VL1 and VL2 paired with VH-variants VH1-VH4 yielded improved stability upon their long-term (83 days) storage compared to the reference C0132 comprising the previously humanized anti-HSG
15 sequences of hz679.

Figure 4 shows the stability of newly humanized anti-oxMIF x anti-HSG bispecific Fab-scFv-Fc mAbs compared to C0132 mAbs comprising previously humanized anti-HSG sequences. Stability was assessed by SEC before (day 0) and after storage for 83 days at -80°C (A) or 4°C (B). To allow direct comparison of the samples, values for %
20 monomer were normalized to day 0 (= 100%) for each antibody.

Example 6: Binding of bispecific anti-oxMIF x anti-HSG mAbs to immobilized HSG.

Material and Methods: Streptavidin diluted to 1 µg/mL in PBS was immobilized
25 into 96-well Maxisorb flat-bottom plates (Thermo Fisher Scientific) for 4 hr at room temperature. After blocking with 2% fish gelatine (FG) in TBST overnight at 4°C and washing with TBST, plates were incubated with 100 µL per well of Biotin-PEG₄-dTyr-dLys (HSG)-dGlu-dLys(HSG)-NH₂ (Eurogentec) at 0.1 µg/mL for 1.5 hours at room temperature. After washing with TBST, 100 µL of serial dilutions of bispecific anti-oxMIF
30 x anti-HSG mAbs or control mAbs were added, and plates were incubated with mAbs for 1.5 hours at room temperature. After washing with TBST, bound antibodies were detected using a goat anti-human IgG (Fc-specific)-HRP conjugate and tetramethylbenzidine (TMB) as substrate. The chromogenic reaction was stopped with

3M H₂SO₄ and OD was measured at 450 nm. EC₅₀ values were determined by 4-parameter logistic fit using GraphPad Prism.

Results and Conclusion: The binding of the bispecific anti-oxMIF x anti-HSG mAbs towards immobilized HSG was measured over a broad range of concentrations and the results are illustrated in **Figure 5**. The binding curves (**Figure 5A**) and the calculated EC₅₀ values representing the apparent K_D clearly show that all bispecific Fab-scFv-Fc mAbs (**Figure 5B**, C0176, C0181, C0182, C0186, and C0192) as well as the CrossMabs (**Figure 5C**, C0238, C0239, C0240, C0241, C0245, C0250) comprising newly humanized anti-HSG sequences have either improved or retained affinity to HSG (evidenced by the lower EC₅₀ values), when compared to the reference mAbs C0132 and C0255 having the previously humanized anti-HSG sequences of hz679.

Figure 5 shows the binding of the bispecific anti-oxMIF x anti-HSG mAbs to immobilized HSG. OD values at 450 nm (mean ± SEM, n=3) were plotted against mAb concentrations, and curve fitting was done by 4-parameter logistic fit using GraphPad Prism. (A) binding curve for Fab-scFv-Fc BsMAbs C0132, C0176, C0181, C0182, C0186, C0192 to HSG; (B) EC₅₀ values (mean ± SEM, n=3) of A; (C) EC₅₀ values (mean ± SEM, n=3) for binding of CrossMabs (C0255, C0238, C0239, C0240, C0241, C0245, C0250) to HSG. Dotted line represents the EC₅₀ value for of reference bispecific anti-oxMIF x anti-HSG antibodies.

Example 7: Preserved binding to oxMIF of anti-oxMIF x anti-HSG bispecific antibodies having newly humanized anti-HSG sequence variants VH1-VH4 paired with VL1-VL2.

Material and Methods: Recombinant human MIF diluted in PBS at 1 µg/mL was immobilized into ELISA plates overnight at 4°C (transforming MIF to oxMIF according to Thiele et al., 2015). After blocking, serial dilutions of anti-oxMIF x anti-HSG bispecific antibodies were added to the plates. Finally, bound antibodies were detected using a goat anti-human IgG (Fc-specific)-HRP conjugate and tetramethylbenzidine (TMB) as substrate. The chromogenic reaction was stopped with 3M H₂SO₄ and OD was measured at 450 nm. EC₅₀ values representing the apparent K_D were determined by 4-parameter logistic fit using GraphPad Prism, and the mean (n=2 or 3) ± SEM is shown.

Results and conclusion: The binding of anti-oxMIF x anti-HSG bispecific mAbs towards immobilized MIF (oxMIF) was measured over a broad range of concentrations and the binding curves as well as calculated EC₅₀ values are illustrated in **Figure 6**.

Figure 6 clearly shows that all the anti-oxMIF x anti-HSG mAbs in both formats, Fab-scFv-Fc (**Figure 6A, B**) and CrossMab (**Figure 6C**) - retained their low nanomolar affinity to oxMIF. As a control the bivalent monospecific antibody C0008 (imalumab, reference anti-oxMIF antibody) was used. It is evident that in this setup avidity has an important contribution to oxMIF binding, as C0008 showed stronger interaction with oxMIF compared to the monovalent anti-HSG/oxMIF bispecific antibodies. In summary, neither the use of newly humanized VH/VL sequences for the anti-HSG arm of the bsMabs, nor the choice of the bispecific mAb format (Fab-scFv-Fc *versus* CrossMab) impaired the binding of the mAbs to oxMIF.

Figure 6 shows the preserved binding of anti-oxMIF x anti-HSG bispecific mAbs towards immobilized MIF (oxMIF). Anti-oxMIF x anti-HSG Fab-scFv-Fc mAbs (A, B) and anti- CrossMabs (C) were bound to immobilized oxMIF and were detected by goat anti-human-IgG (Fc-specific)-HRP conjugate and binding curves for Fab-scFv-Fc bsAbs (A) and EC₅₀ values (B-C) are shown (mean $\pm$ SEM, n=2-3). C0008 (imalumab) was used as reference anti-oxMIF mAb.

Example 8: Evaluation of off-target binding of anti-oxMIF x anti-HSG bsMAbs to A2780 MIF knock out cells.

Material and Methods: A2780 MIF ^{-/-} cell line was generated by CRISPR/Cas9 gene editing of human MIF gene in A2780 ovarian carcinoma cell line (ECACC/Sigma # 93112519). In brief, target gene sequence was analyzed, and target sites were located according to the general rules of designing a targeting guidance RNA (gRNA) for GenCRISPR™ system. A guide RNA (gRNA) was designed to specifically recognize the 5' region of the MIF gene (TTGGTGTTTACGATGAACATCGG, SEQ ID NO: 62) and the gRNA sequence was cloned into the PX459 (addgene) vector containing S. pyogenes Cas9 (SpCas9) nuclease. A2780 cells were transiently transfected by electroporation and were plated in 96-well plates by limit dilution to generate isogenic single clones. Isogenic single clones, where the endogenous MIF gene was efficiently mutated, resulting in consequential reduction (or removal) of the expression of the MIF protein were identified by Sanger sequencing screening. The final clone showed a deletion of 10 bp at position +2 after the start codon of the human MIF gene. Absence of endogenous human MIF protein in the A2780 MIF ^{-/-} cell line was confirmed by Western blotting using polyclonal anti-human MIF antibodies.

A2780 MIF^{-/-} cells were dislodged from flask using TrypLE™, washed with PBS and stained with fixable viability dye eFluor780 (diluted 1:2000 in PBS) for 15 min at 4°C. Afterwards, cells were washed with staining buffer (PBS+5% FBS), resuspended at 4x10⁶ cells/ml in staining buffer and plated into 96-well U-bottom plate at 2x10⁵ cells per well/ 50 µL per well. Serial dilutions (2x stocks) of bispecific anti-oxMIF x anti-HSG mAbs C0176, C0181, C0182, C0186, C0192, or the control mAb rituximab (final concentrations 75 nM, 37.5 nM, and 18 nM) were added in 50 µL per well. After incubating for 40 min at 4°C, cells were washed with staining buffer, and resuspended in 100 µL of secondary antibody (goat anti-human IgG (H+L)-AlexaFluor 488 (AF488), diluted 1:100). After incubating for 40 min at 4°C, cells were washed with staining buffer, resuspended in staining buffer and measured on the Cytoflex-S flow cytometer (Beckman Coulter): 10.000 events were acquired after gating on viable (eFluor 780 negative) and data were analyzed with FlowJo. Geometric mean fluorescence intensity (MFI) values for AF488 of viable (eFluor780 negative) cells was plotted against antibody concentration in GraphPad Prism.

Results and Conclusion: **Figure 7** illustrates that bispecific anti-oxMIF x anti-HSG mAbs having the newly humanized anti-HSG sequences VL1 and VL2 paired with VH1-VH4 (C0176, C0181, C0186, C0182, C0192) do not show any relevant off-target binding above rituximab, suggesting a favorable safety profile, as rituximab is being used as therapeutic antibody for many years. Rituximab, was shown to exhibit some surface hydrophobicity, described to result in unspecific binding to cells (Goyon AA et al., 2017, Jain T. et al., 2017). Here rituximab, was uses as a control antibody targeting CD20, a B-cell surface protein which is absent on A2780 cells.

Figure 7 specifically shows the evaluation of off-target binding of anti-oxMIF x anti-HSG bsMAbs to A2780 MIF knock out^{-/-} cells. A2780 MIF^{-/-} cells were stained with serial dilutions of anti-oxMIF x anti-HSG bispecific mAbs C0176, C0181, C0182, C0186, C0192, and control therapeutic mAb rituximab. Binding was detected with AF488-conjugated goat anti-human IgG (H+L) secondary antibody on viable cells. Geometric mean fluorescence intensity (MFI) values for AF488 channel were plotted against mAb concentrations in GraphPad Prism. Dotted line represents the staining (MFI) of secondary antibody only.

Example 9: Thermal stability of newly humanized anti-oxMIF x anti-HSG CrossMAbs.

Thermal stability of newly humanized anti-oxMIF x anti-HSG CrossMabs containing the sequences of the newly humanized VL-variants VL1 and VL2 paired with VH-variants VH1-VH3 (C0238, C0239, C0240, C0241, C0245, and C0250) was evaluated in a head-to-head comparison to a reference anti-oxMIF x anti-HSG CrossMab C0255 having the VH and VL sequences of the previously humanized anti-HSG Ab hz679.

Material and Methods: The thermal stability of antibodies was assessed by nanoscale Differential Scanning Fluorimetry, nanoDSF (**Figure 8**). Purified antibodies formulated in 50 mM HEPES, 50 mM NaCl (pH 7.2) at 0.5 mg/mL were analyzed in quadruplicates in a Prometheus NT.48 device (NanoTemper Technologies). Glass capillaries were filled with 10 μ L of antibody sample, placed into the sample holder and the temperature was increased from 20 to 95°C at a rate of 1°C/min. Simultaneously, the fluorescence intensity at 330 nm and 350 nm was monitored (excitation wavelength 280 nm). Using the manufacturer's software, the ratio of emission intensities (350 nm/330 nm) was plotted as a function of temperature, its first derivative was calculated and the temperature of the inflection point (T_{IP} , °C) of the first unfolding transition determined.

Results and Conclusion: As illustrated in the **Figure 8**, when compared to the C0255 CrossMab comprising the previously humanized anti-HSG sequence, all the CrossMabs comprising newly humanized anti-HSG sequences (C0238, C0240, C0241, C0245, C0250) demonstrated superior stability against thermal unfolding, evidenced from the higher temperature at which the first unfolding transition occurs, which is a measure of overall stability. In summary, CrossMabs containing the newly humanized VL-variants VL1 and VL2 paired with VH-variants VH1-VH3 yielded improved thermal stability compared to the reference C0255 comprising the previously humanized anti-HSG sequences of hz679.

Figure 8 shows the temperature of the inflection point (T_{IP} , °C) of the first unfolding transition of the newly humanized anti-oxMIF x anti-HSG CrossMabs compared to C0255 CrossMab comprising previously humanized anti-HSG sequences. Data represent the mean values ($n=4 \pm$ SD).

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CLAIMS

1. A bispecific anti-tumor antigen/anti-HSG antibody, comprising at least one binding site specifically recognizing a tumor antigen and a binding site specifically recognizing HSG, comprising

- 5 - a light chain variable (VL) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 17, 19, 89, 90, and 91, or
- a light chain variable (VL) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 17, 19, 89, 90, and 91 with one or two further amino acid substitutions and with glutamine at position 100 (Q100), glutamic acid at position
- 10 105 (E105) and isoleucine at position 106 (I106) according to Kabat numbering, and
- a heavy chain variable (VH) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10, o
- a heavy chain variable (VH) domain comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 6, 8, and 10 with one or two further amino acid
- 15 substitutions and with arginine at position 19 (R19) according to Kabat numbering.

2. The antibody of claim 1, comprising a single-chain variable fragment (scFv) specifically recognizing HSG of the formula selected from the group consisting of VH-linker-VL-linker, VL-linker-VH-linker, wherein the linker comprises SEQ ID NO 39 or SEQ

20 ID NO 42.

3. The antibody of claim 1, comprising VL and VH domains specifically recognizing HSG, wherein the VL domain is C-terminally connected to SEQ ID NO 30, and the VH domain is C-terminally connected to SEQ ID NO 38.

25

4. The antibody of any one of claims 1 to 3, comprising a Fc region having SEQ ID 31 comprising asymmetric mutations in each CH3 domain enabling heterodimerization of two CHs from different antibodies, specifically one CH comprises „knob“ mutations T366W and S354C and one CH comprises „hole“ mutations T366S, L368A, Y407V and Y349C according to EU numbering index.

30

5. The antibody of any one of claims 1 to 4, comprising a variant Fc region with reduced or eliminated effector functions and/or FcRn binding, specifically a variant Fc region having amino acid substitutions at any one or more of positions E233, L234, L235,

G236, G237, P238, I253, D265, S267, H268, N297, S298, T299, H310, E318, L328, P329, A330, P331, H435 of SEQ ID 31 according to EU numbering index, and optionally an aglycosylated Fc region.

5 6. The antibody of any one of claims 1 to 5, wherein the anti-tumor antigen is selected from the group consisting of oxMIF, Mesothelin (MSLN), and Folate Receptor alpha (FR α), specifically the anti-tumor antigen is oxMIF.

7. The antibody of claim 6, wherein the binding site specifically recognizing
10 oxMIF comprises,

- a light chain variable domain comprising SEQ ID NO: 27, or a light chain variable domain comprising SEQ ID NO: 27 and further comprising amino acid substitutions M30L and/or P80S, and
- a heavy chain variable domain comprising SEQ ID NO: 46, specifically with
15 amino acid substitutions L5Q and/or W97Y,
wherein the amino acid positions are numbered according to Kabat.

8. The antibody of any one of claims 1 to 7, selected from the group consisting of Fab-scFv-Fc, CrossMab, (scFv)₂-Fc, scFv/scFv-Fc, Fab/(scFv)₂-Fc, Fab/Fab-scFv-Fc
20 Fc (= IgG-central scFv), Fab/Fab-crossFab-Fc, Fab/crossFab-Fc, and IgG-scFv, IgG-(scFv)₂.

9. The antibody of any one of claims 1 to 8 for use in the treatment or detection of malignancies, wherein said antibody is administered to a subject in a first step and a
25 HSG moiety is administered in a second step, wherein said HSG moiety binds to the antibody.

10. The antibody for use of claim 9, wherein said HSG moiety is conjugated to or labeled with one or more diagnostic and/or therapeutic agents, specifically said HSG
30 moiety comprises one or more HSG haptens, one or more diagnostic and/or therapeutic agents, and a chelator.

11. The antibody of any one of claims 1 to 8, wherein said antibody is bound to a HSG moiety conjugated to or labeled with one or more diagnostic and/or therapeutic

agents, specifically said HSG moiety comprises one or more HSG haptens, one or more diagnostic and/or therapeutic agents, and a chelator, specifically the therapeutic agent, is a radionuclide or a cytotoxic drug; and the diagnostic agent is a radionuclide.

5 12. The antibody of claim 11, wherein the chelator binds a radionuclide and is specifically selected from DOTA, DTPA, deferoxamine B (DFO) and DFO*, specifically the radionuclide is selected from the group consisting of ^{11}C , ^{13}N , ^{15}O , ^{18}F , ^{32}P , ^{33}P , ^{47}Sc , ^{59}Fe , ^{64}Cu , ^{67}Cu , ^{67}Ga , ^{75}Se , ^{77}As , ^{89}Sr , ^{89}Zr , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{99}Mo , ^{103}Pd , ^{105}Rh , ^{109}Pd , ^{111}Ag , ^{111}In , ^{123}I , ^{124}I , ^{125}I , ^{131}I , ^{140}La , ^{142}Pr , ^{143}Pr , ^{149}Tb , ^{149}Pm , ^{153}Sm , ^{159}Gd , ^{161}Tb , ^{165}Dy ,
10 ^{166}Dy , ^{166}Ho , ^{169}Yb , ^{169}Er , ^{175}Yb , ^{177}Lu , ^{186}Re , ^{188}Re , ^{189}Re , ^{192}Ir , $^{193\text{m}}\text{Pt}$, $^{195\text{m}}\text{Pt}$, ^{194}Ir , ^{198}Au , ^{199}Au , ^{211}At , ^{211}Pb , ^{212}Pb , ^{212}Bi , ^{213}Bi , ^{211}At , ^{223}Ra , ^{225}Ac , and ^{227}Th .

13. Use of the antibody of any one of claims 1 to 8, in the preparation of a medicament.

15

14. A pharmaceutical composition comprising the antibody of any one of claims 1 to 8 together with a pharmaceutical excipient, specifically the pharmaceutical composition is formulated for intravenous administration.

20 15. The pharmaceutical composition of claim 14 for use in the treatment of a patient suffering from cancer, specifically the treatment of solid tumors, more specifically the treatment of colorectal cancer, ovarian cancer, breast cancer, prostate cancer, pancreas cancer, and lung cancer.