Affimers as an alternative to antibodies for protein biomarker enrichment

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ABSTRACT

Introduction: Assessing the specificity of protein binders is an essential first step in protein biomarker assay development. Affimers are novel protein binders and can potentially replace antibodies in multiple protein capture-based assays. Affimers are selected for their high specificity against the target protein and have benefits over antibodies like batch-to-batch reproducibility and are stable across a wide range of chemical conditions. Here we mimicked a typical initial screening of affimers and commercially available monoclonal antibodies against two non-related proteins, IL-37b and proinsulin, to assess the potential of affimers as alternative to antibodies.
Methods: Binding specificity of anti-IL-37b and anti-proinsulin affimers and antibodies was investigated via magnetic bead-based capture of their recombinant protein targets in human plasma. Captured proteins were analyzed using SDS-PAGE, Coomassie blue staining, Western blotting and LC-MS/MS-based proteomics.
Results: All affimers and antibodies were able to bind their target protein in human plasma. Gel and LC-MS/MS analysis showed that both affimer and antibody-based captures resulted in co-purified background proteins. However, affimer-based captures showed the highest relative enrichment of IL-37b and proinsulin.
Conclusions: For both proteins tested, affimers show higher specificity in purifying their target proteins from human plasma compared to monoclonal antibodies. These results indicate that affimers are promising antibody-replacement tools for protein biomarker assay development.

1. Introduction

Advances in analytical and molecular technologies have yielded a significant amount of novel biomarkers that contributed to understanding molecular mechanisms of health and disease [1]. Of these biomarkers, proteins are of special interest since they are the functional output of the genome and reflect the biological state of cells, tissues and organs under specific conditions at specific time points [2–5]. Proteins are also widely measured in blood plasma for diagnostic purposes [6]. The collection of all proteins in blood plasma, the plasma proteome, is exceptionally challenging to analyze because of the complexity and high dynamic range [7]. Analysis of low abundant proteins for diagnostics can therefore be difficult as their concentrations are an estimated 10^{10}-fold difference compared to the plasma concentrations of the most abundant plasma protein albumin (35–50 mg/mL) that sums up to approximately 55% of the total plasma proteome [7–9].

Analyzing low abundant protein biomarkers is typically performed using protein binders, such as antibodies, to purify proteins from plasma and reduce interfering signals of high abundant proteins or specifically detect only the target protein. Establishing and verifying high affinity and specificity of an antibody to the target protein is an imperative but also time-consuming step in protein biomarker assay development, with no guarantee of success. This is particularly challenging for proteins that can carry multiple post-translational modifications (PTMs). Besides protein expression, the presence of a specific or a set of PTM(s) might have biological relevance and be even more suitable biomarkers. It may be necessary to develop multiple antibodies that specifically detect the presence or absence of specific PTMs, rather than only detecting the protein itself. In practice this is rarely feasible. The use of mass spectrometry (MS) to analyze proteins and their PTMs is an attractive alternative but requires specific high affinity binders to enrich the low abundant plasma proteins [10–13].

Antibodies have a wide application range and are used in a multitude of laboratory protein assays *e.g.*, Western blotting, ELISA, immunoprecipitations (IP) and immunohistochemistry [14]. However, the use of antibodies has several limitations. Antibodies might cross-react with other proteins besides their target protein [15–18], can show poor reproducibility due to batch-to-batch variability [19], experimental conditions may alter a protein's folding and thereby limit antibody binding [20] and the specific epitope of commercial antibodies is often not known or published. In the past decades, multiple alternatives to antibodies have been developed, including affibodies [21], aptamers [22], single-chain variable fragments [23] and Affimer® reagents (from here on referred to as affimer) [24]. Of these, affimers are small and stable recombinant proteins that are based on a stable protein scaffold, derived from the cysteine protease inhibitor family of cystatins, which function in nature as cysteine protease inhibitors. The scaffold contains a four-stranded β sheet and an α helix containing two variable binding loops each containing nine amino acids that can be replaced with alternative randomized sequences which is used to generate affimer libraries of ~10^{10} clones [5,25]. This allows screening of large quantities of protein-binding molecules for specific selection of high affinity binders of the target protein. Importantly, cross-reactivity to proteins related to the target can be minimized via counter-selection. Affimer proteins are generated from bacterial expression systems [25] and can therefore easily be produced in large quantities with minimal batch-to-batch variation. Collectively, these characteristics make affimers suitable to readily replace antibodies in a variety of applications [26].

In this study, we aimed to investigate the binding specificity of affimers compared to antibodies generated against interleukin 37 (IL-37) and proinsulin as two model proteins. IL-37 is one of the latest discovered cytokines of the IL-1 family and has anti-inflammatory properties [27]. The IL-37 gene consists of 6 exons and exists in five splice variants yielding five different IL-37 isoforms, *viz.* IL-37a – e [27,28] (Fig. 1A). Exons 4–6 encode the 12 β-strands that are necessary for the IL-1-like β-trefoil secondary structure [27,28]. Full length IL-37b, with the N-terminal sequence encoded by exons 1 and 2, is the best characterized isoform. In mammalian cells, a naturally processed IL-37b isoform (Val.46-Asp.218), encoded by exons 4–6, was detected after N-terminal cleavage of full length IL-37b [29]. In addition, the full length IL-37b isoform was found to be active *in vivo* and *in vitro* [30]. It is therefore considered that IL-37b is the biological functional isoform [27]. IL-37a and IL-37d are encoded, like IL-37b, by exons 4–6 and might be biologically functional, however there is no evidence of natural processing of these two isoforms. IL37c and IL-37e lack exon 4, which encodes for the first three β-strands and are therefore unlikely to be functional due to predicted misfolding of the protein [28]. To distinguish the various isoforms of IL-37 in biological samples, binding tools are needed with high specificity, not only among the five isoforms but also in relation to other non-related proteins. Commercially available anti-IL-37 antibodies, monoclonal or polyclonal, are specified to bind the full length IL-37b protein [31,32]. Considering the overlapping amino acid sequences across the IL-37-isoforms, it might be challenging for antibodies to specifically distinguish between the IL-37 isoforms.

Our second model protein, proinsulin, is an important prohormone precursor in the synthesis of insulin that is highly relevant for blood glucose homeostasis. Insulin mRNA is translated into the preproinsulin protein that contains a signal peptide for translocation across the endoplasmic reticulum membrane where signal peptidases generate proinsulin [33,34]. Proinsulin is a single chain precursor of insulin, which consists of the insulin A-chain and B-chain connected by the C-peptide. Upon proteolytical cleavage at the B–C and A-C junctions by prohormone convertase-1 and 2, proinsulin is converted into insulin and the C-peptide [34–36] (Fig. 1B). Insulin consists of two peptides, the A and B-chain, which are linked by disulfide bridges [35]. The structural difference of proinsulin compared to insulin is limited to the presence of the C-peptide in between the insulin A and B-chain. The connection site at the B–C and A-C junctions of proinsulin are the only unique sites with respect to insulin and C-peptide. To study the synthesis and metabolism of proinsulin, a specific and efficient binder that differentiates between insulin proteoforms is therefore needed. Commercially available anti-proinsulin antibodies are monoclonal and predominantly raised against the amino acid sequence of full-length proinsulin or sometimes against the B–C and A-C junction [37,38]. On the other hand, anti-insulin and C-peptide antibodies are raised against the amino acid sequence of the A, B-chain or the C-peptide respectively so they could, theoretically, also bind proinsulin.

The small differences in amino acid sequences of IL-37b, compared to its isoforms, and of proinsulin compared to its cleavage products, set a challenge for specific protein binding where affimer may provide an advantage over antibodies. Affimers can be particular specific in detecting proteins that are active or inactive [39] and can even distinguish between proteins that are very similar as is demonstrated for mouse IgG2b and IgG2a [40]. These benefits may prove useful to distinguish the biologically active IL-37b from other isoforms as well as the overlap in amino acid sequence between proinsulin, insulin and the C-peptide. In addition, affimers can be formatted to have a cysteine residue allowing maleimide-based chemistry and subsequent oriented immobilization on a wide variety of surfaces, which results in specific detection of target proteins from complex biological solutions [41,42].

We here describe our comparison of binding specificity of anti-IL-37 and anti-proinsulin affimers and antibodies to their target recombinant proteins aiming to evaluate the efficiency and specificity of target protein binding. We assessed the antibody's and affimer's binding specificities by magnetic bead-based captures, which is a classical method to purify proteins from complex mixtures [43]. These routine-based screenings were conducted with minimal experimental optimization and thus following manufacturer's guidelines as a first step in binder selection for biomarker assay validation. In this work, we assess affimer-based captures by Western blotting and shotgun proteomics that show the potential of affimers for improved specific enrichment of proteins from human plasma.

2. Methods

2.1. Affimer binders

All affimer proteins were generated by Avacta Life Sciences (Wetherby, United Kingdom). The selection and generation of affimer candidates has been previously described [24]. First, the recombinant target proteins were biotinylated using EZ-Link NHS-LC-Biotin (Thermo Fisher Scientific, Massachusetts, United States) according to the manufacturer's guidelines. Subsequently, affimer candidates against the target proteins were selected. For IL-37b, Avacta®'s consensus plant cystatin scaffold phage display library was selected against biotinylated recombinant human IL-37b protein from R&D Systems (Minneapolis, United States; catalog no. 1975-IL-025 and 7585-IL-025/CF, respectively). To generate affimer candidates against proinsulin, the library was selected against biotinylated recombinant human proinsulin (R&D Systems), with counter-selection performed against insulin and the C-peptide. Affimer candidates from the selections were screened for binding toward the associated biotinylated recombinant proteins immobilized to MultiCyt QBeads DevScreen SH (Sartorius, Goettingen, Germany), functionalized with streptavidin according to the manufacturer's guidelines, in a multiplexed assay using an IntelliCyt® iQue Screener (Sartorius, Goettingen, Germany) in 0.2% BSA containing PBS. Binding by anti-IL-37 affimer candidates was detected using an anti-His-PE conjugate (Abcam, Cambridge, United Kingdom), while binding by anti-proinsulin affimer candidates was detected using an anti-HA-488 conjugate (BioLegend, San Diego, California, United States).

Affimer proteins were supplied in PBS, 0.02% sodium azide, pH 7.4 (1 mg/mL). Affimer proteins formatted with a cysteine residue at the C-terminus were biotinylated via the sulfhydryl group of the cysteine using EZ-Link Maleimide-PEG$_{11}$ (Thermo Fisher Scientific) according to
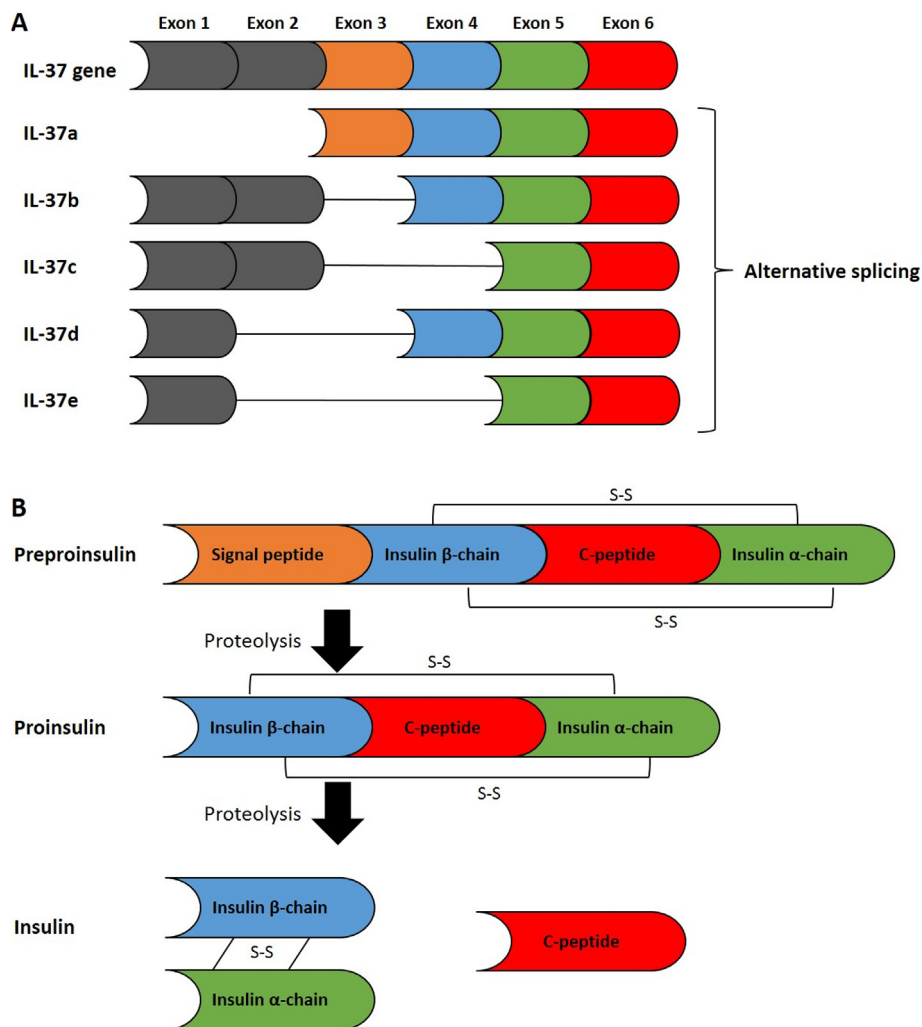
Fig. 1. (A) Graphical overview of alternative splicing of the IL-37 gene that yields different IL-37 protein isoforms and **(B)** the synthesis of preproinsulin into proinsulin by signal peptidases, which then is cleaved into insulin by prohormone convertase-1 and 2 at the B-chain - C-peptide and A-chain - C-peptide junctions.

the manufacturer's guidelines.

2.2. Affinity enrichment with antibodies and affimers

2.2.1. Antibody-based capture

Protein G Dynabeads (1 mg) (Invitrogen, Carlsbad, United States) were coupled to 1 μg monoclonal anti-IL-37 or anti-proinsulin antibody, IL-37 and PI mAb, respectively, (R&D Systems and Abcam, respectively). The capture was performed according to the manufacturer's guideline deliberately without any optimizations. In short: The antibody-coupled beads were washed thrice, using PBS-Tween 0.05%, pH 7.4, and incubated with 500 ng of recombinant IL-37b or proinsulin spiked in human blood plasma from a healthy individual (covered by informed consent). After magnetic separation, beads were washed three times. The bound fraction was eluted from the antibody-bead complex by adding 50 mM glycine pH 2.8 followed by pH neutralization with 1/10th volume of 1 M Tris HCl, pH 8 and then mixed with SDS sample buffer in a ratio of 3:1 (v/v) eluted fraction: SDS sample buffer. The SDS sample buffer consisted of 2% SDS and 10% 2-mercaptoethanol. The mixture was incubated at 70 °C for 10 min. Subsequently, the captured proteins were purified by removal of debris and beads through magnetic separation after centrifugation at 2900 x g at 4 °C for 2 min. Supernatants were directly analyzed by Western blotting. As a control experiment, 1 mg of protein G magnetic beads was coupled to 1 μg mouse anti-GAPDH antibody (Abcam) (for mock purification) and

subsequently incubated in plasma. Further handlings of the experimental procedure were identical as written above.

2.2.2. Affimer-based capture

A total of 10 μg biotinylated anti-IL-37 affimers were bound to 500 μg Dynabeads MyOne streptavidin T1 magnetic beads (Invitrogen) and 10 μg anti-proinsulin affimers were bound to an equivalent binding capacity amount (50 μg) MagReSyn streptavidin beads (ReSyn Biosciences, Edenvale, South Africa). The capture was performed according to Avacta®'s guidelines deliberately without any optimizations. In short: The affimer-bead complex was washed twice (1x with 50 μL 50 mM sodium phosphate + 300 mM sodium chloride 0.05% Tween-20, pH 8 and 1x with 50 μL PBS, pH 7.4), and incubated with 500 ng of recombinant IL-37 or proinsulin spiked in human blood plasma from a healthy individual (covered by informed consent). Next, the affimer-bead complex was magnetically separated and washed five times. Finally, the bound fraction was eluted by incubation with SDS sample buffer at 95 °C for 5 min. Subsequently, the captured proteins were purified by removal of debris and beads through magnetic separation after centrifugation at 2900 x g at 4 °C for 2 min. Supernatants were directly analyzed by Western blotting. As a control experiment, 500 μg of Dynabeads MyOne streptavidin T1 magnetic beads (Invitrogen) were incubated in plasma. Further handlings of the experimental procedure were identical as written above.

2.3. Protein staining and Western blot analysis following SDS-PAGE electrophoresis

Captured proteins were separated by SDS-PAGE and visualized by Coomassie blue staining and Western blotting. For sample preparation, the samples were loaded on a 10% or 15% polyacrylamide SDS-PAGE gel for IL-37b or proinsulin-related captures, respectively, followed by electrophoresis under reducing conditions. Subsequently, gels were stained with colloidal Coomassie blue (Severn Biotech Ltd., Worcestershire, United Kingdom) on a rocking shaker overnight. The gel was destained with fresh 30% methanol:MilliQ every hour until the protein bands were visible against the background.

For Western blotting, proteins were transferred from SDS-PAGE onto a PVDF membrane and blocked for 1 h at room temperature with 3% (w/v) BSA in TBS/Tween 20. The membrane was incubated with goat anti-IL-37 IgG polyclonal antibody (R&D Systems), or mouse anti-proinsulin IgG mAb (Abcam) (1:2000 with 3% (w/v) BSA in TBS/Tween 20) at room temperature for 2 h. Next, the membrane was washed and incubated with a donkey-anti goat IgG antibody (Sigma-Aldrich, Saint Louis, United States) (1:2500) or a goat-anti mouse antibody (Dako, Glostrup, Denmark) (1:5000) (diluted in 5% (w/v) milk powder in TBS/Tween 20) for 2 h at room temperature. Protein bands were developed with super enhanced chemiluminescence (sECL) (Thermo Fisher Scientific) and visualized with Chemidoc XRS+ (Biorad, Hercules, USA) using Image Lab software (Biorad).

2.4. In gel digestion for bottom-up proteomics

The in-gel digestion protocol was performed essentially as described by Shevchenko A. et al. [44] with minor modifications. Briefly, entire lanes of interest were cut out of the SDS-PAGE gels and protein bands at ~25 and 50 kDa, corresponding with light and heavy chains from immunoglobulins or anti-IL-37/proinsulin antibodies, were removed. The remainder of the gel lane was chopped into 1 × 1 mm pieces approximately and submitted to in-gel digestion. Gel pieces were incubated in 50 mM ammonium bicarbonate (Sigma-Aldrich) for 5 min. For reduction of disulfide bonds, samples were incubated with 10 mM dithiothreitol (Fluka, Buchs, Switzerland) for 20 min at 56 °C followed by alkylation by incubating with 50 mM 2-chloroacetamide (Fluka) in the dark at room temperature. Digestion was performed with sequencing grade-modified trypsin (Promega, Mannheim, Germany) overnight at 37 °C. Tryptic digestion was quenched by adding 2% trifluoroacetic acid to an end concentration of 1%, where after the supernatant was collected and the procedure repeated with 0.1% formic acid in acetonitrile instead of 2% trifluoroacetic acid. The resulting supernatant was pooled and concentrated to an end volume of 2 μL with a Concentrator plus (Eppendorf, Hamburg, Germany) at room temperature.

Next, samples were desalted and concentrated with 100 μL Bond Elut OMIX C18 tips (Agilent, Santa Clara, United states) according to the protocol provided by the manufacturer with small buffer adjustments. Finally, samples were concentrated to a volume of 2 μL with a Concentrator plus (Eppendorf) removing all acetonitrile. Subsequently, 0.1% formic acid was added to an end volume of 20 μL after which samples were used for mass spectrometry (MS) analysis.

2.5. Bottom-up proteomics

Bottom-up proteomics experiments were performed using ultra high pressure nano flow liquid chromatography (nano-Advance; Bruker Daltonics, Bremen, Germany) coupled online to an ultra-high resolution quadrupole time-of-flight mass spectrometer (maXis Plus; Bruker Daltonics) via an axial desolvation vacuum assisted electrospray ionization source (Captive sprayer; Bruker Daltonics). Tryptic digests were loaded onto the C18 reversed phase trapping column (Acclaim PepMap C18 column 3 μm particles, 100 Å pore-size, 75 μm internal diameter × 20 mm length; Thermo Fisher Scientific) at 7 μL/min using

0.1% acetic acid. Peptides were separated on a C18 reversed phase analytical column (Acclaim PepMap RSLC C18 column, 2 μm particles, 100 Å pore-size, 75 μm internal diameter × 15 cm length; Thermo Fisher Scientific) using a linear gradient of 3–40% acetonitrile in 0.1% acetic acid within 30 min at a flow rate of 500 nL/min at 45 °C. The Q-ToF instrument operated in positive ion mode with optimized tuning for ions up to 1500 m/z Data-dependent MS/MS spectra acquisition (AutoMSn mode) was performed using the following parameters: preferred charge states: 2–4, ignore singly charged ions, allow unknown charge states, exclude ions < 350 m/z, MS + MS/MS cycle time: 3 sec, dynamic exclusion enabled for 30 sec, allow precursor ion re-selection if current intensity/previous intensity > 4. Internal mass calibration was performed using sodium acetate clusters introduced at the beginning of each analysis via direct infusion of 0.2% (w/v) sodium acetate in 50% 2-propanol. For protein annotation, raw data were imported in Maxquant software [45] version 1.6.0.16 for database searches using the human UniProt FASTA database (version June 2019). The following parameters were in MaxQuant: Trypsin - specific cleavage was used as enzyme mode with a maximum 2 missed cleavages was allowed. Carbamidomethyl (Cys) as fixed modification and methionine oxidation and N-terminal acetylation as variable modifications were used for protein quantitation. False discovery rate (FDR) was set at 0.01 for proteins and a minimum length of 6 amino acids for peptides was used. FDR was determined by searching spectra against a decoy database. Only proteins identified by two or more peptides were used for further analysis and MS peak intensity values (from here on referred to as MS signals) were used to evaluate protein abundances. Keratins and trypsin were considered as contaminants and therefore deleted from the list with identified proteins. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [46] partner repository with the dataset identifier PXD016435.

3. Results

We determined the binding specificity of anti-IL-37 and anti-proinsulin affimers and antibodies in two steps. We first investigated the efficiency and specificity of affimers and antibodies to purify spiked recombinant IL-37b and proinsulin in human plasma using Coomassie stained SDS-PAGE or Western blotting. Next, we analyzed the protein composition of all capture experiments by shotgun proteomics.

3.1. Generation of highly specific affimers

Following phage display selection using recombinant IL-37a and IL-37b, nine affimer candidates were identified as specific for recombinant IL-37b during screening assays by Avacta Life Sciences (Supplementary Fig. 1A). Similarly, two affimer candidates, selected for using recombinant proinsulin were identified as specific for their respective targets during screening (Supplementary Fig. 1B). Affimer candidates were screened for cross-reactivity toward five control proteins and showed negligible binding (Supplementary Fig. 1). We selected anti-IL-37 affimer candidate E8 and anti-proinsulin affimer candidate 2851 for comparisons with the antibody-based captures.

3.2. Gel-based analysis of protein binding specificities

Affimer and antibody-based captures on recombinant IL-37b and proinsulin spiked in human plasma were conducted. We have not further optimized the experimental conditions as suggested by the vendor as we intended to mimic the fast screening of protein binders that researchers would do as an initial step prior to further optimization of the selected binder. After SDS-PAGE, the captured protein fraction was stained by Coomassie blue to visualize the total protein complement bound by affimers and antibodies, or by Western blot analysis to confirm if the spiked recombinant protein was indeed captured.

Fig. 2A shows the Coomassie blue staining of affimer E8 and mAb-
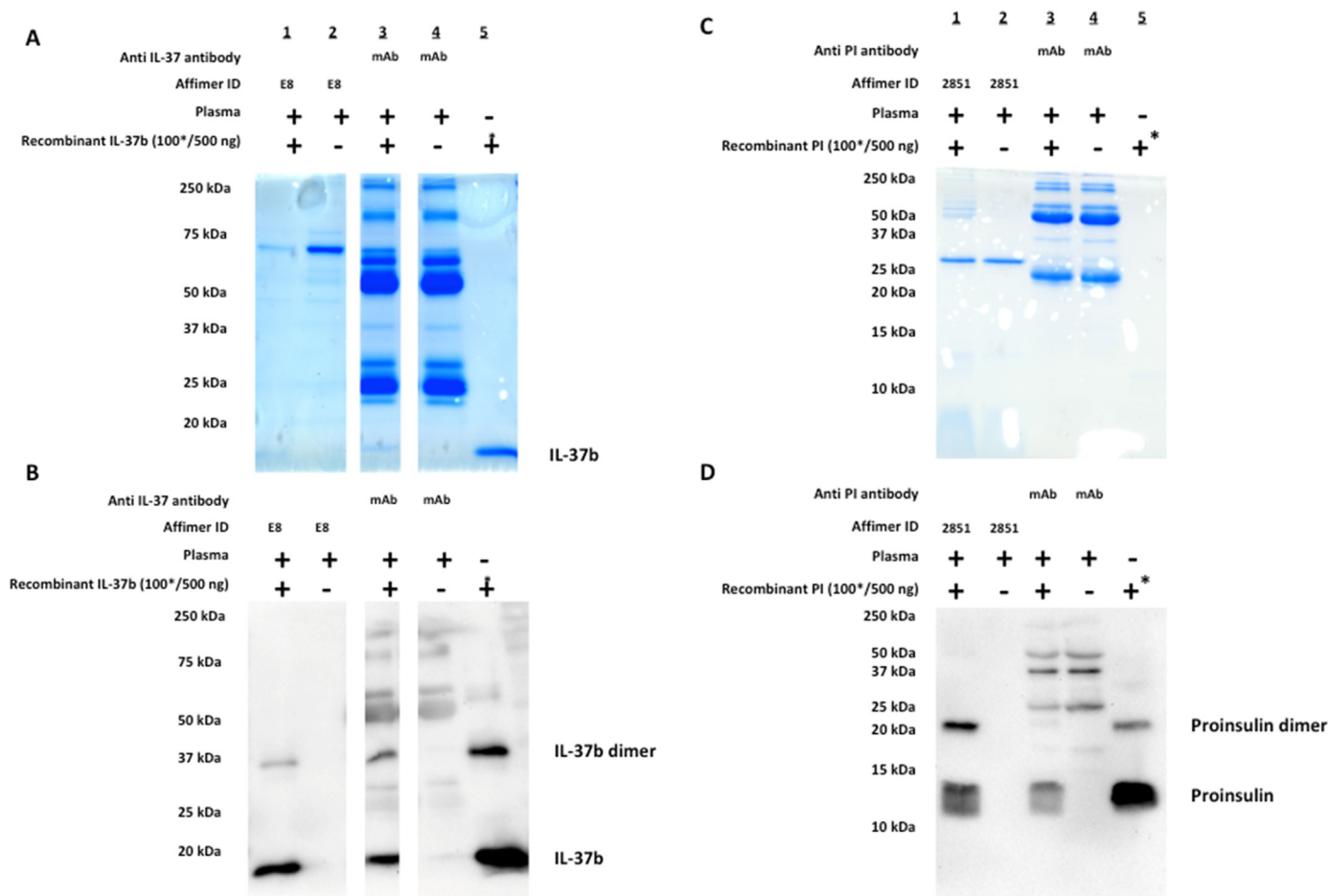
Fig. 2. Coomassie blue staining **(A)** and Western blot analysis **(B)** of fractions from an affimer-based capture of recombinant IL-37b spiked in plasma and control plasma, lanes 1 and 2 respectively, and an antibody-based capture on recombinant IL-37b spiked in plasma and control plasma, lanes 3 and 4 respectively. Lane 5 depicts recombinant IL-37b as positive control. Coomassie blue staining **(C)** and Western blot analysis **(D)** of fractions from an affimer-based capture on recombinant proinsulin (PI) spiked in plasma and control plasma, lanes 1 and 2 respectively. An antibody-based capture on recombinant proinsulin spiked in plasma and control plasma is shown in lanes 3 and 4 respectively. Lane 5 depicts recombinant proinsulin as positive control. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

based captures (lanes 1 and 3, respectively) of recombinant IL-37b spiked in plasma. A weak band at ~19 kDa was observed in lane 1 and 3 corresponding to the positive control recombinant IL-37b sample (calculated mass 19.4 kDa) in lane 5 of Fig. 2A. In addition, the Western blot analysis confirmed the identity of the 19 kDa band in lanes 1 and 3 (Fig. 2B). A possible dimer of recombinant IL-37b was observed at ~37 kDa in lanes 1, 3 and 5 (Fig. 2B).

Fig. 2C shows the Coomassie blue staining of affimer 2851 and mAb-based captures (lanes 1 and 3, respectively) for recombinant proinsulin spiked in plasma. We did not observe an expected protein band at ~10 kDa in lanes 1, 3 and 5 (Fig. 2C) corresponding to re-combinant proinsulin (calculated mass 10.5 kDa) possibly due to the detection limit of the protein staining. However, the Western blot analysis confirmed the presence of recombinant proinsulin (lanes 1, 3 and 5, Fig. 2D) at the expected molecular weight of recombinant proinsulin. A possible dimer of recombinant proinsulin was observed at ~20 kDa in lanes 1, 3 and 7, Fig. 2D.

In addition to the specific capture of their spiked target recombinant proteins by the anti-IL-37 and anti-proinsulin affimers and antibodies, we also observed other protein bands in the gel analyses. To further investigate this, we also conducted the captures on control plasma without spiked recombinant proteins to evaluate co-purified back-ground plasma proteins.

Coomassie blue staining of anti-IL-37 affimer E8 (Fig. 2A, lanes 1 and 2) and anti-proinsulin affimer 2851-based captures (Fig. 2C, lanes 1

and 2) revealed small amounts of protein bands between ~50 and 200 kDa. Anti-IL-37 affimer E8-based capture revealed an additional abundant protein band at ~65 kDa (lane 2, Fig. 2A). Anti-proinsulin affimer2851-based capture revealed additional protein bands at ~26 kDa in lanes 1 and 2 (Fig. 2C). These bands may be co-eluted anti-proinsulin affimers (~26 kDa, lanes 1 and 2, Fig. 2C), whereas we did not observe the same bands for anti-IL-37 affimers (lanes 1 and 2, Fig. 2A). In contrast, Coomassie staining of anti-IL-37 mAb-based cap-ture (Fig. 2A, lanes 3 and 4) and anti-proinsulin mAb-based capture (Fig. 2C, lanes 3 and 4) showed more protein bands in number and abundance at ~37 kDa and between ~50 and 200 kDa. In addition, all antibody-based captures showed intense protein bands at ~25 and 50 kDa, likely showing the mAb's light and heavy chains eluted from the protein G beads. Alternatively, these protein bands could also ori-ginate from plasma-resident immunoglobulins that bound to the protein G beads during the experimental procedures. Potentially, the protein bands at ~150–250 kDa could reflect intact antibody IgGs due to in-complete reduction. In general, gel-based analyses showed that affimer-based captures resulted in less additional purified proteins then anti-body-based captures.

3.3. Mass spectrometry-based analysis of protein binding specificities

Next, we performed bottom-up proteomics to characterize the pro-tein composition of the affimer and antibody-based captures. This was

done by an in-gel digestion of the purified proteins (lanes 1–4, Fig. 2A and C). After evaluation of detected background proteins derived from the affimer and antibody-based capture experiments, we determined the intensity of IL-37b and proinsulin relative to other proteins to evaluate the binding specificity of respective affimers and antibodies. This approach consisted of multiple steps which will be delineated below.

3.3.1. Analysis of background proteins

The use of streptavidin or protein G beads may result in different interactions with proteins from plasma. To investigate which proteins in either purification system (streptavidin and protein G respectively) were co-purified as background, we performed mock purifications. For the protein G beads, we first incubated the beads with anti-GAPDH antibody to coat the beads with a non-related antibody and used this to investigate common protein G background. Dynabeads MyOne streptavidin T1 beads (streptavidin background) and protein G beads – anti GAPDH complex (protein G background) were then incubated with human plasma to identify which proteins could be considered as background signals. The identified proteins from these controls were compared with those identified from the affimer and antibody based-captures to determine specificity of binding. We considered all proteins that overlapped between the streptavidin background and affimer-coated streptavidin beads captures as background proteins, as well as proteins that overlapped between the protein G background and protein G-coated anti-IL-37b or PI mAb captures (indicated in Fig. 3A/C, 4A/C in orange).

For affimer-based IL-37b captures, we identified in total 40 background proteins consisting of 25 proteins identified in all captures and an additional fifteen overlapping proteins between the streptavidin background and affimer-coated streptavidin beads capture in plasma without spiking IL-37b (Fig. 3A). For affimer-based proinsulin captures, we identified a total of 28 background proteins consisting of nineteen proteins that were identified in all captures, eight proteins that were identified in both the streptavidin background and the affimer-coated streptavidin beads capture of plasma without spiking proinsulin. One additional protein was identified that overlapped between the streptavidin background and affimer-coated streptavidin beads capture of plasma with spiked proinsulin (Fig. 4A). In addition, for the proinsulin-related affimer captures we independently determined and compared the background proteins from magnetic streptavidin beads of two different vendors. All ide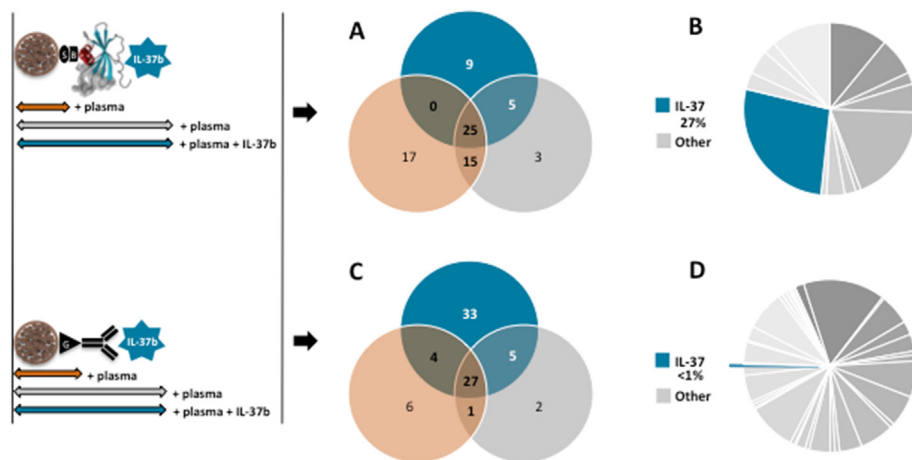ntified background proteins were evaluated in a protein abundance database (PaxDB) [47] and belonged to the most abundant proteins from human plasma, except streptavidin (Supplementary Table 1). The use of magnetic streptavidin beads, independent from which vendor, with and without an immobilized affimer, resulted in co-purified non-related high abundant plasma proteins.

The same evaluation for background plasma proteins from the protein G magnetic bead-based capture experiments was conducted. For antibody-based IL-37b captures, a total of 32 background proteins were identified of which 27 were observed in all captures and four background proteins between antibody-coated protein G beads capture of plasma with spiked IL-37b and the protein G background. One additional protein was identified that overlapped between the protein G background and antibody-coated protein G beads capture of plasma without spiked proinsulin (Fig. 3C). For antibody-based proinsulin captures, 24 background proteins were identified of which nine were observed in all captures and fifteen overlapped between antibody-coated protein G beads capture of plasma without spiked proinsulin and the protein G background (Fig. 4C). All identified proteins belonged to the most abundant proteins from human plasma, except calmodulin-like protein 5 (Supplementary Table 1). Similar to magnetic streptavidin beads, the use of protein G magnetic beads resulted in co-purified non-related high abundant plasma proteins.

3.3.2. IL-37b and proinsulin abundance

Anti-IL-37 affimer and mAb-based captures of spiked recombinant IL-37b and control plasma resulted in a total of seventeen and 40 purified proteins, respectively (Fig. 3A and C). IL-37b could only be detected in the plasma spiked with recombinant IL-37b. To evaluate the abundance of IL-37b compared to other co-purified proteins, we compared the MS signals from all captured proteins except the observed background proteins that overlapped with the streptavidin bead or protein G bead background of the affimer and mAb-based captures of spiked recombinant IL-37b, which were 14 and 38 proteins respectively (numbers in white, Fig. 3A and C). IL-37b was the most abundant protein in the affimer-based capture as it accounted for 27% of the total MS-signal from all fourteen proteins derived from the affimer capture experiments (Fig. 3B) whereas less than 1% of the total MS-signal from all 38 proteins accounted for IL-37b derived from the mAb capture experiments (Fig. 3D). Supplementary Table 2 provides protein identities and MS signals of all detected proteins depicted in Fig. 3A and C.

For proinsulin similar analyses were performed. Anti-proinsulin

Fig. 3. Graphical illustration of the affimer and antibody-based capture experiments of recombinant IL-37b. Number of proteins identified from streptavidin (upper image) or protein G bead (lower image)-based capture of control plasma are indicted in orange. Number of proteins identified from the affimer (upper image) and antibody (lower image)-based captures on control plasma and spiked recombinant IL-37b are indicated in gray and blue, respectively. (A) Venn diagrams depicting the number of identified proteins from affimer E8-based captures on plasma spiked with recombinant IL-37b and control plasma as well as proteins identified from streptavidin bead background. (B) Relative abundance of the IL-37b signal to the total mass spectrometry signal of proteins identified from affimer E8-based capture of spiked IL-37b and the overlapping proteins from affimer E8-based capture of control plasma (white numbers, figure A). (C) Venn diagrams depicting the number of identified proteins from monoclonal antibody (mAb)-based captures on plasma spiked with recombinant IL-37b and control plasma as well as proteins identified from protein G bead background. (D) Relative abundance of the IL-37b signal to the total mass spectrometry signal of proteins identified from mAb-based capture of spiked IL-37b and the overlapping proteins from mAb-based capture of control plasma (white number, figure C). Upper magnetic bead represents streptavidin (S) coating and the immobilization of a biotinylated (B) affimer. The lower magnetic bead represents protein G (G) coating and the linkage to the Fc and Fab region of the monoclonal antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
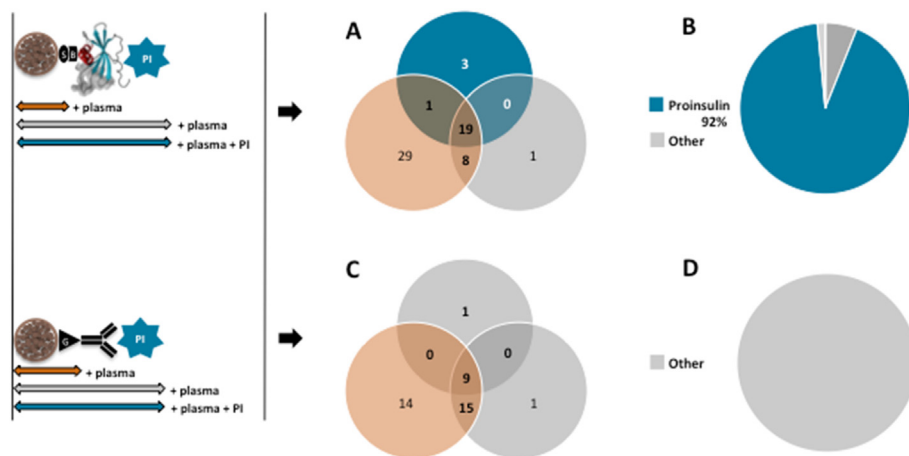
Fig. 4. Graphical illustration of the affimer and antibody-based capture experiments of recombinant proinsulin. Number of proteins identified from streptavidin (upper image) or protein G bead (lower image)-based capture of control plasma are indicted in orange. Number of proteins identified from the affimer (upper image) and antibody (lower image)-based captures on control plasma and spiked recombinant proinsulin are indicated in gray and blue, respectively. **(A)** Venn diagrams depicting the number of identified proteins from affimer 2851-based captures on plasma spiked with recombinant proinsulin and control plasma as well as proteins identified from streptavidin bead background. **(B)** Relative abundance of the proinsulin signal to the total mass spectrometry signal of proteins identified from affimer 2851-based capture of spiked proinsulin and the overlapping proteins from affimer 2851-based capture of control plasma (white numbers, figure A). **(C)** Venn diagrams depicting the number of identified proteins from monoclonal antibody (mAb)-based captures on plasma spiked with recombinant proinsulin and control plasma as well as proteins identified from protein G bead background. **(D)** Relative abundance of the proinsulin signal to the total mass spectrometry signal of proteins identified from mAb-based capture of spiked proinsulin and the overlapping proteins from mAb-based capture of control plasma (figure C). Upper magnetic bead represents streptavidin (S) coating and the immobilization of a biotinylated (B) affimer. The lower magnetic bead represents protein G (G) coating and the linkage to the Fc and Fab region of the monoclonal antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

affimer and mAb-based captures of spiked recombinant proinsulin and control plasma resulted in four and two purified proteins, respectively (Fig. 4A and C). Proinsulin was only detected in the affimer-based capture on proinsulin spiked in plasma (white numbers, Fig. 4A). Subsequently, proinsulin's abundance compared to other co-purified proteins was determined by evaluating the protein MS signals corresponding to the captured proteins from the affimer and mAb capture experiments. Proinsulin was the most abundant protein as it accounted for 92% of the total MS-signal of all three proteins (Fig. 4B). Only one proinsulin-related peptide was detected from the mAb-based capture of spiked recombinant proinsulin (Fig. 4D), which did not meet our criteria of at least two peptides for identification (data not shown). Supplementary Table 3 provides protein identities and MS signals of all proteins specifically detected from the affimer and antibody-based captures on the respective spiked recombinant target protein as depicted in Fig. 4A and C. In general, the affimer-based captures purified their target protein from a complex matrix more efficiently relative to the background proteins compared to respective antibody-based captures.

4. Discussion

Protein purification is a key methodology to analyze protein biomarkers for monitoring health and disease, enabling development of novel diagnostic tests. Antibodies are widely used as affinity binders for protein enrichment upstream of immune-based or mass spectrometry-based biomarker analyses. However, antibodies can be variable in specificity and/or selectivity in binding their target protein. Freedman et al. (2015) [48] have estimated that about 36% of the errors contributing to irreproducibility are caused by the biological reagents. Therefore, affinity binders need a proper specificity-validation before they are implemented for the development of new protein capture assays. In recent years, several alternative protein binders with potential to replace antibodies have been developed, including affimers [24]. In the current study, we demonstrate that affimers show better purification properties of their target proteins relative to antibodies for two selected proteins, using gel-based and mass spectrometry-based analyses.

Previously, others [41,49–53] demonstrated that affimers have great specificity to their target protein. This study adds another head-to-head comparison of affimers and antibodies against the same target protein as was shown earlier by Weckman et al. (2016) [40]. However,

we here present a clear improvement of protein purification using affimers. There is an unmet need for validation of any affinity protein binder, *i.e.* antibody, affimer or other to determine their specificity and reproducibility in human plasma, as this is highly relevant for diagnostics [54,55]. There are several publications with recommendations for antibody validation [10,56–58]. The U.S. Food and Drug Administration claims that, during assay validation, antibodies should be appropriately characterized by evaluating binding properties and cross-reactivity [59]. In line with this, the Structural Genomics Consortium has defined three key parameters that characterizes whether an affinity binder is specific: 1) the target protein should be captured, 2) the relative abundance of the target protein should be in the top three compared to co-immunopurified a-specific proteins and 3) the affinity binder also captures the endogenous protein in its native environment [58]. Moreover, since its launch in 2005 the Human Protein Atlas [60] has contributed significantly by providing a continuously growing database on antibody validations based on tissue and cellular specific localization of corresponding human proteins. In addition, the immense number of commercially available antibodies is usually offered with a credible validation report. However, these validation reports often lack supportive data for diagnostic assay development and are therefore insufficient. Therefore, in any case, it is recommended to screen the specificity of a protein binder prior to protein biomarker assay development.

Here, we conducted an initial screening to assess binding specificities of commercially available antibodies and generated affimers using manufacturer's guidelines for magnetic bead-based capture experiments. Our approach was to characterize enriched fractions by Coomassie blue staining, Western blot and LC-MS proteomics. Our results indicated that all affimers and antibodies tested in this study were able to bind their target protein. However, the affimers proved their advantage over antibodies to capture the cognate protein more specifically from plasma irrespective of the number of co-purified background proteins and magnetic streptavidin beads used from a different vendor. All of these background proteins, except for two, are among the most abundant plasma proteins, including albumin, which is a known carrier of many different protein and lipoproteins [61]. In addition, pI values of plasma proteins close to that of albumin are likely to be co-precipitated during downstream analysis [62]. Earlier work by Zhou et al. (2004) [61] demonstrated that a proteomic profiling of purified high abundant plasma proteins revealed a vast network of associated protein-protein and protein-peptide interactions in which 210 proteins

were identified that were co-purified with these six high abundant plasma proteins. Comparable to our current study, this study by Zhou et al. (2004) [61] identified 53 proteins from a protein G bead-capture experiment of human serum. It is therefore plausible to suggest that the observed background in this study relates to protein interactions with the high abundant plasma proteins which are incidentally co-purified, illustrating the need for high selectivity in affinity protein binding to maximize the detection of relevant protein biomarkers.

Without any adjustments to the manufacturer's guidelines, the high specificity and affinity of affimers to their target protein as demonstrated in this study highlights their potential as attractive antibody-replacement tool. The identification of an optimal affimer binder is based on a phage display system, not limited by an animal host's immune system, whereas affimer protein production can be strongly upscaled in recombinant biotechnological approaches, ensuring rapid and high batch-to-batch reproducibility [24,25,63]. This provides a unique advantage of affimers to recognize conformational epitopes whereas antibodies prefer to recognize linear amino acid sequences [39]. In addition, binding properties of affimers are less affected by alterations in experimental conditions due to their high stability. These advantages enable specific protein purification strategies that are less likely to be successful when using antibodies. For instance, affimers are stable in a wide pH and temperature [24,26] range, potentially enabling protein binding that cannot be considered using antibodies. The affimer scaffold has a melting point in excess of 100 °C [24] enabling protein binding under denaturing conditions if the epitope in the protein's native form is inaccessible or hidden due to protein aggregation. In addition, hidden epitopes in protein complexes or due to protein-protein interactions may become accessible when exposed to low pH conditions in which an affimer can still bind its target protein. Collectively, affimers are interesting antibody-replacement tools in different protein affinity purification assays *e.g.*, ELISA, IPs, multiplex assays and immunohistochemistry. However, further studies are needed to validate and compare binding characteristics of affimers and antibodies. We believe when experimental conditions and materials are similar and optimized for both antibody-based and affimer-based captures, the affimer's performance will prove its advantage even more over antibodies. In addition, future studies should also address and compare affimer and antibody affinities since this is another critical parameter of affinity binders for diagnostic purposes.

5. Conclusions

We show that analyzing capture experiments with gel-based Coomassie staining and Western blotting and MS-based proteomics provides valuable data on the binding characteristics of affimers and antibodies. In our study, affimers had a more efficient purification of their target proteins from human plasma as exemplary complex matrix. This study contributes to our understanding that affimers are promising alternatives for antibodies in protein affinity purification assays.

CRediT authorship contribution statement

Roel Tans: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Danique M.H. van Rijswijck:** Formal analysis, Investigation. **Alex Davidson:** Validation, Resources. **Ryan Hannam:** Validation, Resources. **Bryon Ricketts:** Validation, Resources. **Cees J. Tack:** Writing - review & editing. **Hans J.C.T. Wessels:** Writing - review & editing, Visualization. **Jolein Gloerich:** Writing - review & editing, Visualization, Project administration. **Alain J. van Gool:** Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2020.105677.

Declarations of interest

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