The Trimeric Autotransporter Adhesin YadA of Yersinia enterocolitica Serotype O:9 Binds Glycan Moieties

Ina Meuskens1, Juan Leva-Bueno2, Paul Millner2, Monika Schütz3, Sally A. Peyman4 and Dirk Linke1*

1 Section for Genetics and Evolutionary Biology, Department of Biosciences, University of Oslo, Oslo, Norway, 2 Faculty of Biological Sciences, School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom, 3 Interfakultäres Institut für Mikrobiologie und Infektionsmedizin Tübingen (IMIT), Institut für Medizinische Mikrobiologie und Hygiene, Universität Tübingen, Tübingen, Germany, 4 Molecular and Nanoscale Physics Group, Department of Physics and Astronomy, University of Leeds, Leeds, United Kingdom

Yersinia adhesin A (YadA) is a key virulence factor of Yersinia enterocolitica and Yersinia pseudotuberculosis. YadA is a trimeric autotransporter adhesin, a class of adhesins that have been shown to enable many Gram-negative pathogens to adhere to/interact with the host extracellular matrix proteins such as collagen, vitronectin, and fibronectin. Here, we show for the first time that YadA of Yersinia enterocolitica serotype O:9 not only interacts with proteinaceous surface molecules but can also attach directly to glycan moieties. We show that YadA from Y. enterocolitica serotype O:9 does not interact with the vitronectin protein itself but exclusively with its N-linked glycans. We also show that YadA can target other glycan moieties as found in heparin, for example. So far, little is known about specific interactions between bacterial autotransporter adhesins and glycans. This could potentially lead to new antimicrobial treatment strategies, as well as diagnostic applications.

Keywords: trimeric autotransporter adhesin, bacterial adhesion, virulence, extracellular matrix (ECM), adhesion, glycan

INTRODUCTION

Yersinia adhesin A (YadA), a type Vc trimeric autotransporter adhesin of Yersinia spp. is crucial for virulence. YadA is encoded on a virulence plasmid, the pYV plasmid. Expression of the YadA gene is temperature controlled, and upon a temperature shift to 37°C, once the bacterium enters the host, the expression of YadA is initiated (Toivanen and Skurnik, 1992).

YadA is a surface-exposed adhesin that is anchored in the bacterial outer membrane via a trimeric β-barrel domain (Shahid et al., 2015). The passenger domain of YadA, a trimeric coiled-coil stalk, and an N-terminal β-roll head domain are channeled through the barrel in an unfolded state during the autotransport process (Chauhan et al., 2019). Upon autotransport, the passenger domain starts folding, building a rigid structure protruding toward the outside of the cell (Chauhan et al., 2019). Here, the head domain has been shown to be responsible for many of YadAs adhesion properties (Leo et al., 2008; Mühlenkamp et al., 2015).
While YadA is typically classified as an adhesin that aids in pathogen–host interactions via interactions with the extracellular matrix (ECM) (Tamm et al., 1993; Westerlund and Korhonen, 1993; Leo et al., 2008; Keller et al., 2015), YadA has also been shown to be involved in immune evasion (Tamm et al., 1993; Westerlund and Korhonen, 1993; Grosskinsky et al., 2007; Leo et al., 2008; Schindler et al., 2012; Keller et al., 2015). YadA knockout mutants are avirulent (Pepe et al., 1995; Schütz et al., 2010). This is only partially due to the adhesion properties of YadA as *Yersinia* spp. have additional adhesins which can replace its function (Mallick et al., 2012; Chauhan et al., 2016). During an infection with *Yersinia enterocolitica*, YadA is involved in surface adhesion and has been shown to interact with a variety of proteinaceous ECM molecules such as collagen, fibronectin, and vitronectin (Vn) (Tertti et al., 1992; Schulze-Koops et al., 1993; Leo et al., 2008; Mühlenkamp et al., 2017). The interaction with ECM varies in strength and depends on environmental shear forces (Müller et al., 2011). While the YadA head domain is conserved among *Yersinia* species, some *Y. enterocolitica* strains and *Yersinia pseudotuberculosis* exhibit an additional stretch of approximately 31 residues at the N-terminus of each monomer of the YadA head domain (Figure 1A). This stretch has been shown to be responsible for Vn binding (Mühlenkamp et al., 2017).

Vn has been described as an incidental component of the ECM (Leavesley et al., 2013). The ECM is a matrix composed of a variety of proteins, such as collagen, fibronectin, and laminin, and also proteoglycans and glycosaminoglycans (GAGs) forming a hydrogel. This matrix surrounds cellular components of the cell surface and provides strength and elasticity (Frantz et al., 2010). Vn is an approximately 75 kDa glycoprotein involved in tissue repair. It is heavily glycosylated, exhibiting three N-linked glycans (N86, N169, and N242) (Hwang et al., 2014). Vn shows great flexibility, and its conformational state is greatly dependent on its glycosylations. The ability of Vn to associate with GAGs on interaction partners such as heparin (Izumi et al., 1989; Stockmann et al., 1993). The ability of Vn to associate with GAGs like heparin and heparan sulfate, which are in turn part of the ECM, contributes to the function of Vn in tissue repair (Leavesley et al., 2013). The ECM and its components are an attractive binding target for *Y. enterocolitica* and *Y. pseudotuberculosis* as surface adhesion is crucial for subsequent tissue invasion (Pepe et al., 1995).

Here, we report that YadA from *Y. enterocolitica* strain E40, serotype O:9 (YadA<sub>O:9</sub>) interacts with Vn via its glycosylations. We furthermore show that YadA<sub>O:9</sub> can directly interact with heparin. Up until now, an interaction with glycan moieties like the glycosylations of ECM proteins or GAGs has not been described for YadA.

### Bacterial Strains and Growth Conditions

Bacteria were cultivated in Lysogeny broth (LB, Miller formulation). For whole-cell assays with fluorescence detection, *Escherichia coli* Top10 glmS:sGFP (AS75) was used and grown in the presence of arabinoose (Saragliadis and Linke, 2019). For protein purification, genes encoding the proteins of interest were expressed in *E. coli* BL21 (DE3) Gold. Generally, bacteria were grown at 37°C to the desired OD<sub>600</sub>. During overexpression, the temperature was shifted to 23°C after induction.

### YadA Head Domain Purification

pASK-IBA3 YadA<sub>O:8/O:9</sub> was transformed into *E. coli* BL21 (DE3) Gold and grown on ampicillin plates. A single colony was inoculated into 20 ml of LB medium supplemented with 100 µg/ml ampicillin and grown at 37°C overnight (o/n). The following day, a 2 L subculture was prepared and grown in a home-built fermentation system (a system where air is bubbling through bottles of growth medium that stand in a temperature-controlled water bath) until an OD<sub>600</sub> of 0.5–0.7 was reached. The temperature was shifted to 23°C, and expression was induced with 0.2 µg/ml anhydrotetracycline (AHTC). Protein expression was allowed for 16 h. The culture was harvested by centrifugation at 4,000 × g. Afterward, the pellet was resuspended in Tris-buffered saline (TBS) buffer (20 mM Tris pH 7.5, 300 mM NaCl, 20 mM imidazole) with 8 µg/ml lysozyme and a pinch of DNase. The suspension was subjected to cell lysis using a French press after addition of a HALT protease inhibitor mix (1:500, Thermo Fisher Scientific; 1861278). The lysate was centrifuged for 1 h at 69,600 × g, and the supernatant was then filtered through a 0.2 µm filter and subjected to Ni-NTA affinity chromatography (Cytiva, 17531901). As YadA with C-terminal His<sub>6</sub>-tag elutes at high imidazole concentrations (160–500 mM), the protein was pure enough for binding experiments after Ni-affinity chromatography. The protein was subjected to dialysis against TBS buffer (20 mM Tris pH 7.5, 150 mM NaCl).

### Vitronectin Binding Experiments With Whole Bacteria

*E. coli* AS75 with pASK-iba4C YadA<sub>O:8</sub> or pASK-iba4C YadA<sub>O:9</sub> was grown o/n in LB medium supplemented with 0.02% (w/v) arabinoise and 100 µg/ml ampicillin. The next day, the cultures were diluted 1:100 in 20 ml LB medium supplemented as before and grown at 37°C to an OD<sub>600</sub> of 0.5. YadA expression was then induced by the addition of AHTC to a final concentration of 0.2 µg/ml and grown for another 3 h at 37°C. YadA expression was checked for by visual inspection for auto-aggregation. In the meantime, clear flat-bottom 96-well plates were coated with 100 µl of a 10 µg/ml Vn solution, from either plasma (Gibco, PHE0011), recombinantly expressed in HEK cell cultures (Merck/Millipore, SRP3186), or *E. coli* (Thermo Fisher Scientific, A14700), by incubation for 1 h at room temperature (RT). The Vn solution was discarded from the plates, and the wells were washed three times with TBS (20 mM Tris.

### MATERIALS AND METHODS

#### Plasmids and Constructs

Plasmids and constructs used in this study are listed in Table 1, and sequences can be found in the supplements (Table 1). Constructs made in this study were cloned using the Gibson assembly (Gibson et al., 2009).
pASK-IBA3_YadA
pASK-IBA4C_YadA
Bacteria”). Then 100 µl TBS buffer, and fluorescence was measured using an excitation wavelength of 488 nm and recording the emission at 533 nm (BioTek Synergy H). For experiments with deglycosylated Vn, the experiment was performed the same way, but deglycosylated Vn (see section “Deglycosylation of Vitronectin”) was used for coating.

### TABLE 1 | Constructs used in this study.

| Construct                        | Resistance      | Source strain              | Source                           |
|----------------------------------|-----------------|---------------------------|----------------------------------|
| pASK-IBA4C_YadA<sub>O:8</sub>    | Chloramphenicol | Y. enterocolitica O:8 WA-314 | Mühlenkamp et al., 2017          |
| pASK-IBA4C_YadA<sub>O:9</sub>    | Chloramphenicol | Y. enterocolitica O:9 E40   | Mühlenkamp et al., 2017          |
| pASK-IBA3_YadA<sub>O:8</sub> head domain | Ampicillin      | Y. enterocolitica O:8 WA-314 | This study (supplement)          |
| pASK-IBA3_YadA<sub>O:9</sub> head domain | Ampicillin      | Y. enterocolitica O:9 E40   | This study (supplement)          |

Heparin Inhibition Assay Using Microscopy
Glass coverslips were coated with 50 µl Vn (10 µg/ml) at 4°C o/n. An o/n culture of E. coli AS75 harboring pASK-IBA4C_YadA<sub>O:8/O:9</sub> was inoculated into LB supplemented with 20 µg/ml chloramphenicol and 0.02% w/v arabinose. The next day, the culture was diluted 1:100 in the same broth, and the culture was grown to OD<sub>600</sub> of 0.5 followed by induction with 0.2 µg/ml AHTC and yda expression for 3 h at 37°C. In the meantime, Vn-coated coverslips were incubated with TBS or 100 µM heparin-disaccharide I-S (Merck, H9267-1MG) for 1 h at RT where applicable. After that, all coverslips were blocked with 3% (w/v) BSA in TBS for 1 h at RT. One hundred microliters of 5 × 10<sup>8</sup> bacteria in suspension were centrifuged at 4,000 × g for 5 min and resuspended in either TBS (20 mM Tris pH 7.5, 150 mM NaCl) or 100 µM heparin-disaccharide in TBS and incubated for 1 h at RT. After that, the bacteria were centrifuged down again and washed three times in 100 µl TBS. Finally, the bacteria were resuspended in 1 ml 3% BSA in TBS. Three hundred microliters of the bacteria was added to the coverslips and incubated for 30 min at RT. The supernatant was discarded, and the coverslips were washed three times with TBS and fixed with 500 µl of 4% (w/v) paraformaldehyde in TBS for 20 min at RT. Finally, the coverslips were mounted in 5 µl ProLong Glass Antifade Mountant (Invitrogen, P36980) and dried o/n. Microscopy was performed using a fluorescent microscope (Zeiss Axioplan 2) and a 100× oil immersion objective. For quantification, images were converted into binary files, and the area of the particles was calculated using Fiji (Supplementary Figure 3). Mean areas were plotted including the standard error of the mean.

Disaggregation Experiments Using Microscopy
An o/n culture of E. coli AS75 harboring pASK-IBA4C_YadA<sub>O:8/O:9</sub> was inoculated into LB supplemented with 100 µg/ml ampicillin and 0.02% (w/v) arabinose. The next day, the culture was diluted 1:100 in the same broth and grown to OD<sub>600</sub> of 0.5 followed by induction with 0.2 µg/ml AHTC and yda expression for 3 h at 37°C. The culture was diluted to an OD<sub>600</sub> of 1.0, and 50 µl was centrifuged down at 4,000 × g for 5 min. The pellets were then resuspended in 50 µl TBS or TBS supplemented with 100 µM heparin-disaccharide (Merck, H9267-1MG). This was incubated at 37°C in a shaking incubator for 30 min. Five microliters of each solution was wet-mounted onto microscope slides, and the edges were sealed using a CoverGrip coverslip sealant (Biotium, 23005). Microscopy was

Deglycosylation of Vitronectin
For deglycosylation of Vn, 20 µg of Vn from the respective sources (in water) was mixed with 2 µg of the glycopeptidase PNGase F (500 U) (Promega, V4831) and incubated at 37°C for 19 h. The non-deglycosylated control samples of Cn were incubated at 37°C for 19 h, omitting the PNGase F. For PNGase F control samples, 2 µl of PNGase F was added to water and incubated as described before. Successful deglycosylation was checked for on a SDS-PAGE gel with subsequent silver staining (Nesterenko et al., 1994).
performed using a fluorescence microscope (Zeiss Axioplan 2) and a x100 oil immersion objective. For quantification, images were converted into binary files, and the particle sizes were calculated using Fiji (Supplementary Figure 4). The area of each individual particle was plotted in a column scatter plot.

**Dot Blots for Heparin Binding to YadA Head Domains**
Nitrocellulose membranes were cut and transferred into a six-well plate. Three 2 µl drops of a 700 µg/ml purified YadA<sub>0.8</sub> or YadA<sub>0.9</sub> solution were applied onto the membrane and air-dried. Then, the membrane was blocked with 5% BSA in TBS-T (20 mM Tris pH 7.5, 150 mM NaCl) for 1 h at RT. Five hundred microliters of a 100 µM biotinylated heparin (Merck, B9806-10MG) solution in TBS-T was incubated on the membrane for 1 h at RT. The membrane was washed three times with TBS-T and afterward incubated with 500 µl of 1:10,000 diluted Strep-Tactin–HRP conjugate (IBA Lifesciences, 2-1502-001) in 5% BSA in TBS-T for 30 min at RT. After the membrane was washed three times with TBS-T and once with TBS (20 mM Tris pH 7.5, 150 mM NaCl), a 500 µl ECL reagent (Thermo Fisher Scientific, 320106) was added, and the membrane was immediately imaged using a Kodak Image Station 4000R.

**Heparin Binding Assay Using Bacteria**
An o/n culture of E. coli AS75 pASK-IBA4C_YadA<sub>0.8/0.9</sub> was grown in the presence of 0.2% (w/v) arabinose and 100 µg/ml ampicillin. This culture was diluted 1:100 the next morning and grown to an OD<sub>600</sub> of 0.5. YadA full-length expression was induced by addition of 0.2 µg/ml AHTC. Expression was allowed for 3 h at 37°C. Uninduced bacteria were used as a control. The bacteria were diluted to an OD<sub>600</sub> of 0.2, spun down, and resuspended in phosphate-buffered saline (PBS). One hundred microliters of that bacterial solution was pipetted into 96-well plates and centrifuged at 4,000 × g. After that, 100 µl of a 10 µg/ml biotinylated heparin (Merck, B9806-10MG) solution in 3% (w/v) BSA in TBS was added and incubated at RT for 0.5 h. The plate was washed three times with TBS. The plate was centrifuged as before after every wash before discarding the washing buffer. Strep-Tactin–HRP conjugates (IBA Lifesciences, 2-1502-001) at 1:1,000 in 3% (w/v) BSA in TBS were added and incubated for 30 min at RT. The plate was washed as described before. The ABTS solution was prepared, and color development was stopped as described before. Wells that did not contain any bacteria were used as background controls. Absorbance at 405 nm was measured in a plate reader (BioTek Synergy H).

**Binding Assay Using Purified YadA Head Domains**
One hundred microliters of 10 µg/ml YadA in TBS was coated into a 96-well plate by incubation at RT for 1 h. The plate was washed three times with 200 µl TBS (20 mM Tris pH 7.5, 150 mM NaCl) and blocked using 200 µl of 3% BSA in TBS. Afterward, 100 µl of biotinylated heparin dilution (0–6.75 µg/ml) in TBS was added to the wells and incubated for 1 h at RT. The wells were washed three times with TBS as described above and blocked with 3% BSA in TBS for 1 h at RT. Strep-Tactin–HRP (IBA Lifesciences, 2-1502-001) at 1:1,000 was added in 3% BSA in TBS and incubated for 1 h at RT. The wells were washed again as described earlier, and an ABTS solution was used for detection as described before. After color development, the reaction was stopped by adding 100 µl of a 1% SDS solution. Absorbance at 405 nm was measured in a plate reader (BioTek Synergy H).

**Binding Assay Using Immobilized Heparin on Impedimetric Nanobiosensors**
Gold screen-printed electrodes (BVT-AC1.W1.RS.Dw2) from BVT Technologies were employed for biosensor fabrication. The electrodes were pre-treated by washing with 97% v/v ethanol for 30 min, rinsed with deionized water, and dried with N<sub>2</sub>. Twenty-five microliters of 2.5 mM octopamine in 10 mM phosphate buffer pH 7.2 was spread across the working electrode and electro-polymerized for two cycles at a scan rate of 100 mV/s from +0.0 to +1.6 V. The electrodes were rinsed with 10 mM PBS and dried with Ar. The electrodes were functionalized by binding of biotinylated NeutrAvidin (Ahmed et al., 2013). After that, 10 µl of a 1 mg/ml biotinylated heparin was coated onto the surface for 1 h at RT. The surface was washed with 10 mM PBS and dried with Ar.

**Electrochemical Impedance Spectroscopy Measurements and Data Treatment**
For binding measurements, 10 µl of E. coli AS75 expressing either YadA<sub>0.8</sub> or YadA<sub>0.9</sub> full length at OD<sub>600</sub> of 2, 0.2, and 0.02 was applied to the working electrodes and incubated for 30 min at RT. Blanks were acquired by measuring 10 mM phosphate buffer, omitting the bacteria. Electrical impedance measurements were carried out in a three-cell system of a PalmSens4 potentiostat, galvanostat, and frequency response analyzer (PalmSens BV, Netherland), adding 10 mM [Fe(CN)<sub>6</sub>]<sup>3−/4−</sup> in 10 mM PBS pH 7.2 onto the electrodes. EIS measurements were recorded at 0 V over a frequency range of 5–0.1 Hz, with a modulation voltage of + 10 mV. Measurements corresponding to finite Warburg impedances were excluded from the Nyquist plots (Nguyen and Breitkopf, 2018). PSTrace (5.8) was used to record the EIS measurements. Metrohm Autolab Nova 2.1.4. was used to fit the EIS measurements. Meukens et al. YadA Binds to Glycans
Statistical Analysis
For binding data analysis, data are shown as means ± SD and were analyzed using a one-way ANOVA including Tukey’s test. For data plotting and statistical analysis, OriginPro and “R” were used. For microscopy analysis, mean particle areas ± SEM were plotted. As the particles sizes were not normally distributed, non-parametric testing including a Kruskal-Wallis test and subsequent Wilcox testing were applied to test for significance. Significance levels are indicated in the graphs with p < 0.05 (*), p < 0.01 (**), or p < 0.001 (***).

RESULTS
Head Domain of YadA From Y. enterocolitica Serotype O:9 Binds to Vitronectin Isolated From Plasma and HEK Cells but Not to Vitronectin Produced in E. coli
We first aimed to describe the molecular details of the interaction between the YadA head domain of Y. enterocolitica serotype O:9 (YadA<sub>O:9</sub>) and Vn. YadA<sub>O:9</sub> harbors an additional, N-terminal, 31-residue stretch (Figure 1A) that has been described to interact with Vn (Mühlenkamp et al., 2017). We started out replicating the experiments done by Mühlenkamp et al. (2017). For these enzyme-linked immunosorbent assay (ELISA)-like binding experiments, Vn from different sources was used. Vn purified from plasma (Vn<sub>plasma</sub>), Vn expressed in HEK cell culture (Vn<sub>HEK</sub>), and Vn recombinantly expressed in E. coli (Vn<sub>Ec</sub>) were tested for their capacity to be bound by E. coli expressing either full-length YadA<sub>O:8</sub> or YadA<sub>O:9</sub> (Figure 1B). Additionally, Vn binding by purified YadA head domains was tested (Figure 1C). We reasoned that, if YadA indeed bound to a conserved sequence within Vn, the binding should happen irrespective of the origin of Vn and only with YadA<sub>O:9</sub>. Indeed, the whole-cell assays show that only YadA<sub>O:9</sub>-expressing bacteria bound to Vn<sub>plasma</sub> and Vn<sub>HEK</sub> (Figure 1B). Binding between Vn<sub>Ec</sub> and YadA<sub>O:9</sub> could not be observed. Bacteria expressing YadA<sub>O:8</sub> did not bind to either Vn variant (Figure 1B). These findings were corroborated by assays using purified YadA head domains. While we observed some binding of purified YadA<sub>O:8</sub> to Vn<sub>plasma</sub>, no binding to Vn<sub>HEK</sub> and Vn<sub>Ec</sub> was observed (Figure 1C). The weak binding of purified YadA<sub>O:8</sub> can be explained by Vn<sub>plasma</sub> being contaminated with other proteins (Supplementary Figure 1). YadA<sub>O:9</sub> on the other hand showed clear binding to all Vn variants, with reduced binding to Vn<sub>Ec</sub>. Based on these findings, we sought to investigate the difference between YadA<sub>O:9</sub> binding to Vn<sub>plasma</sub> and Vn<sub>HEK</sub> and binding to Vn<sub>Ec</sub>.

FIGURE 1 | YadA<sub>O:9</sub> binds to Vn<sub>plasma</sub> and Vn<sub>HEK</sub>, but shows reduced binding to Vn<sub>Ec</sub>. (A) Alignment of the N-terminal head domains of the YadA head domain from Y. enterocolitica serotypes O:8 and O:9. The alignments include the head domain of YadA, the neck region, and the first 20 residues of the coiled-coil stalk domain. YadA<sub>O:9</sub> has an insertion of 31 residues toward the N-terminus of the head domain. (B) Whole-cell binding assay to Vn using E. coli expressing either YadA<sub>O:8</sub> or YadA<sub>O:9</sub>. (C) Vn binding assay with purified YadA<sub>O:8</sub> and YadA<sub>O:9</sub> head domains. Significance levels are indicated with *p < 0.05, **p < 0.01, ***p < 0.001.
The Head Domain of YadA From Y. enterocolitica Serotype O:9 Only Binds Glycosylated Vitronectin

Due to the observation that YadA<sub>O:9</sub> binds Vn<sub>plasma</sub> and Vn<sub>HEK</sub> but shows at least reduced binding to Vn<sub>Ec</sub>, we wanted to investigate whether YadA<sub>O:9</sub> actually binds a stretch within Vn or whether it either recognized a folded binding site or the glycosylations of Vn. Vn is heavily glycosylated with at least three N-linked glycans at residues N86, N169, and N242 (Figure 2A). As eukaryotic proteins recombinantly expressed in E. coli are usually not glycosylated, we first tested the latter hypothesis. We used PNGase F, a glycopeptidase that selectively removes glycans directly at the N-linkage by cleaving the glycosidic bond between asparagine and the core GlcNAc. With the deglycosylated Vn, the binding assays were repeated to see whether binding could be abrogated by removal of the N-linked glycosylations. In Figure 2B, the fluorescence-based whole-cell assay using E. coli AS75 expressing either full-length YadA<sub>O:8</sub> or full-length YadA<sub>O:9</sub> is shown. No binding was observed with cells expressing YadA<sub>O:8</sub>, which fits the hypothesis, as the postulated Vn binding stretch is not present in YadA from Y. enterocolitica serotype O:8. In the case of binding of bacteria expressing full-length YadA<sub>O:9</sub>, a clear difference in binding to Vn<sub>plasma</sub> was observed between the glycosylated Vn<sub>plasma</sub> and deglycosylated Vn<sub>plasma</sub> (Figure 2B). For Vn<sub>HEK</sub>, no change in binding of YadA<sub>O:9</sub>-expressing E. coli AS75 before and after glycosylation was observed. We can at this point not say as to why no change was observed for bacterial binding of Vn<sub>HEK</sub> compared to deglycosylated Vn<sub>HEK</sub>. Vn<sub>Ec</sub> was bound in neither the glycosylated nor the deglycosylated state. While this supported our hypothesis that the glycan residues of Vn might be involved in the YadA<sub>O:9</sub>–Vn interaction rather than the proteinaceous part of Vn, we also repeated the binding assay using purified YadA head domains from both serotypes of Y. enterocolitica (Figure 2C).

Binding of YadA<sub>O:9</sub> to Heparin Abrogates the YadA<sub>O:9</sub>–Vitronectin Interaction

Heparin was described to abrogate the interaction between Vn and YadA<sub>O:9</sub> (Mühlenkamp et al., 2017). We next wanted to investigate whether the potential YadA<sub>O:9</sub> glycan interaction might be the cause for this observation. It was hypothesized before that heparin blocks the YadA binding site on Vn. As in the globular state, the heparin binding site in Vn is mostly hidden inside the core of the protein; this seemed unlikely to be the reason for YadA<sub>O:9</sub> binding inhibition...
Meuskens et al. YadA Binds to Glycans

FIGURE 3 | Binding of E. coli expressing YadA_0:9 is reduced when the bacteria are preincubated with heparin-disaccharide. Vn from different sources was coated onto coverslips, and binding of fluorescence E. coli expressing YadA_0:9 was measured (left column). Images in the middle column show micrographs of Vn-coated coverslips that were preincubated with 100 µM heparin-disaccharide. Afterward, binding of fluorescent E. coli expressing YadA_0:9 was monitored. When Vn was coated and binding of fluorescent E. coli expressing YadA_0:9 that were preincubated with heparin was (column 3) reduced, adhesion was measured for both Vn_plasma and Vn_HEK. E. coli expressing YadA_0:9 did only weakly bind to Vn_HEK as already observed in previous experiments. In the last column, the mean area of particles (µm²) ± SEM was calculated and plotted as bar graphs. Significance levels are indicated with *p < 0.05, **p < 0.01, ***p < 0.001.

(Hayashi et al., 1985; Izumi et al., 1989; Zhuang et al., 1996; Leavesley et al., 2013). Coverslips were coated with untreated Vn_plasma, Vn_HEK, or Vn_EC. YadA_0:9 (full length)-expressing, fluorescent bacteria were checked for binding (Figure 3, left column). To check for the influence of heparin on this interaction, we also prepared samples where we either preincubated Vn with heparin (Figure 3, middle column) or preincubated YadA_0:9-expressing bacteria with heparin (Figure 3, right column). In the fluorescence microscopy adhesion assay, we observed only minimal adhesion of bacteria to Vn_EC (Figure 3, bottom row). When coverslips had been coated with Vn_plasma or Vn_HEK, adhesion was observed only in the absence of heparin. In cases where Vn was preincubated with heparin, bacteria expressing YadA_0:9 adhered to Vn to a comparable level as in the untreated samples (Figure 3, left and middle columns). When YadA_0:9-expressing bacteria were preincubated with heparin, reduced binding to untreated Vn_plasma and Vn_HEK was observed (Figure 3, right column). Quantifications of the area of the particles reflect the tendencies seen in the experiment, where preincubation of the bacteria expressing YadA_0:9 with heparin seems to reduce binding to Vn whereas preincubation of Vn with heparin did not change the adhesion of YadA_0:9-expressing bacteria. This observation further strengthened our hypothesis that the YadA Vn-binding loop aids in adhesion of YadA_0:9 to glycan moieties.

The Head Domain of YadA From Y. enterocolitica Serotype O:9 Prefers Heparin Binding Over Autoaggregation

YadA, as an adhesin, is involved in autoaggregation, which has been described as an important mechanism for immune evasion during infection as well as for biofilm formation (Trunk et al., 2018). We have observed earlier that the interaction with other adhesin targets, such as ECM molecules, interferes with autoaggregation (manuscript in preparation). We thus wanted to investigate what effect heparin might have on autoaggregation mediated by YadA_0:9. We expressed YadA_0:8 or YadA_0:9 full length in fluorescent E. coli AS75 and allowed for autoaggregation of these samples. Uninduced samples served as a control. Half of the samples were then preincubated with heparin-disaccharide. The uninduced samples did not show any autoaggregation behavior, either in the presence or in the absence of heparin (Figure 4, rows 1 and 3). The induced YadA_0:8 samples autoaggregated to similar degrees both in the absence and in the presence of heparin (Figure 4, row 2). Fluorescent bacteria
expressing YadA$_{O:9}$ clearly showed heparin binding (Figure 5A). To support these results, we used electrochemical impedance measurements to measure bacterial binding to a heparin-coated surface. Biotinylated heparin was coated onto a biosensor using matrix-embedded NeutrAvidin (Ahmed et al., 2013). The change in impedance was then measured upon binding of E. coli AS75 expressing either YadA$_{O:8}$ or YadA$_{O:9}$ (Figure 5B). Please note that negative binding values are due to stronger adhesion of E. coli expressing YadA$_{O:9}$ to uncoated electrodes that were used as a background and subtracted. While, for uninduced E. coli AS75 and E. coli expressing YadA$_{O:8}$, no change in impedance was observed, we could clearly measure binding of E. coli expressing YadA$_{O:9}$ by a significant change of impedance (Figure 5B). We then aimed to test for binding of heparin to purified YadA head domains. A dot blot using immobilized YadA$_{O:8}$ and YadA$_{O:9}$ head domains to detect binding of biotinylated heparin was performed. While no heparin binding was observed for either the buffer control or the YadA$_{O:8}$ head domain, a signal could be observed for the binding of
Meuskens et al. YadA Binds to Glycans

FIGURE 5 | YadA<sub>O:9</sub> directly binds to heparin. (A) Whole-cell binding assay of heparin to E. coli expressing YadA<sub>O:8</sub> or YadA<sub>O:9</sub> shows binding of YadA<sub>O:9</sub>-expressing bacterial binding to heparin. (B) Impedimetric biosensor experiment showing the adhesion of bacteria expressing YadA<sub>O:8</sub> or YadA<sub>O:9</sub> to surface-coated heparin. (C) Dot blot using immobilized YadA<sub>O:8</sub> or YadA<sub>O:9</sub> head domains shows direct binding to biotinylated heparin. (D) ELISA-like binding assay using immobilized YadA head domains to capture biotinylated heparin. (E) ELISA-like binding assay showing the interaction between immobilized, purified YadA head domains and biotinylated heparin in a concentration-dependent manner. Significance levels are indicated with *p < 0.05, **p < 0.01, ***p < 0.001.

biotinylated heparin to the immobilized YadA<sub>O:9</sub> head domain (Figure 5C). To quantify the binding, we immobilized the head domains of YadA<sub>O:8</sub> and YadA<sub>O:9</sub> in a 96-well plate and tested for binding at various concentrations. We observed that at 450 µg/ml of heparin, binding between YadA<sub>O:8</sub> or YadA<sub>O:9</sub> head domains and heparin is significantly different (Figures 5D,E). Repeating the assay with a dilution series of biotinylated heparin allowed us to investigate the concentration dependency of the binding. Using a fifth-party logistics fit, we estimate the (apparent) K<sub>D</sub> to be approximately 30 nM. Furthermore, this experiment allows for an estimation of the binding ratio between YadA<sub>O:9</sub> and biotinylated heparin. The binding ratio is estimated to be 1:1 (YadA<sub>O:9</sub> monomer to biotinylated heparin). We can at this point not claim an accurate K<sub>D</sub> or binding ratio as heparin varies in length but averages at 15 kDa (Shriver et al., 2012).

DISCUSSION

With this work, we present evidence that a 31-residue loop insertion specifically found in YadA<sub>O:9</sub> is responsible for the interaction between YadA<sub>O:9</sub> and glycan moieties. All experiments presented in this work were done using YadA from Y. enterocolitica strains WA-314 (serotype O:8) or E40 (serotype O:9). While Vn binding results published previously indicate that all Y. enterocolitica strains of serotype O:9 harbor this N-terminal 31-residue loop (Mühlenkamp et al., 2017), we cannot be sure that the presence or absence of this loop correlates with the serotypes in all cases. To our knowledge, sequence variations of YadA have never been reported to contribute directly to serotyping.

This loop region aids in the interaction between YadA<sub>O:9</sub> and the glycosylated host protein Vn as well as heparin, which, like Vn, is part of the ECM. We show that this interaction is not specific for one type of glycan residue but rather for a variety of glycans. This is supported by the observation that YadA<sub>O:9</sub> interacts not only with the glycan residues of the glycoprotein Vn but also with the carbohydrate polymer heparin. Interactions with glycans are employed by many pathogens for adhesion and invasion, especially in viruses (Marks et al., 2001; Guan et al., 2017; Sorin et al., 2021). Also, bacterial virulence factors like UpaB and Pili have been shown to interact with the glycosylations of glycoproteins and GAGs (Rajas et al., 2017; Paxman et al., 2019; Sauer et al., 2019; Vizarraga et al., 2021). To our knowledge, this is the first time that a trimeric autotransporter adhesin is described to bind glycans.
While it has been established in earlier work that YadA\textsubscript{O,9} interacts directly with human Vn (Mühlenkamp et al., 2017), we show that recombinant Vn expressed in \textit{E. coli} is not bound by YadA\textsubscript{O,9}. Eukaryotic proteins expressed in \textit{E. coli} often lack glycosylations, as \textit{E. coli} does not possess the glycosyltransferases and glycosidases present in eukaryotes (Sahdev et al., 2008; Khow and Suntrarachun, 2012). We further show in deglycosylation experiments that properly deglycosylated Vn was not bound by YadA\textsubscript{O,9} any longer. This, and the fact that binding does not occur when using YadA\textsubscript{O,8}, further supports our model that the YadA\textsubscript{O,9} loop is responsible for interactions with glycans. Furthermore, as Mühlenkamp et al. (2017) had described that heparin could inhibit the interaction between YadA\textsubscript{O,9} and Vn, we set out to investigate whether heparin binding to Vn was actually the reason for this inhibition or whether a more direct interaction of heparin with YadA\textsubscript{O,9} was the reason for this effect. While Vn indeed harbors a heparin binding site, this site is hidden in globular Vn (Izumi et al., 1989). We thus checked for binding of fluorescent \textit{E. coli} expressing full-length YadA\textsubscript{O,9} after preincubating either Vn with heparin-disaccharide or after preincubating fluorescent \textit{E. coli} expressing YadA\textsubscript{O,9} with heparin-disaccharide. Heparin preincubation of fluorescent \textit{E. coli} expressing YadA\textsubscript{O,9} inhibited Vn interaction, while preincubation of Vn with heparin-disaccharide did not. This is in agreement with literature stating that only 2% of the overall plasma Vn is present in a heparin-binding-competent state (Izumi et al., 1989), as well as with our model stating that the YadA\textsubscript{O,9} 31-aa loop might be responsible for glycan binding. Furthermore, heparin-disaccharide was able to dissolve the YadA-mediated autoaggregation of bacteria expressing YadA\textsubscript{O,9}, which again indicates that there might be a direct interaction between heparin and YadA\textsubscript{O,9}. Finally, we were able to directly show the interaction using YadA\textsubscript{O,9}-expressing \textit{E. coli} as well as purified YadA\textsubscript{O,9} head domains in dot blots and ELISA-like assays. When looking at the YadA\textsubscript{O,9} sequence, one can see that the loop contains seven positively charged residues (Arg and Lys). We hypothesize that the interaction between YadA\textsubscript{O,9} could be explained by charge interactions with these residues, as the terminal sugar of the glycosylation of Vn is in most cases negatively charged sialic acid (Hwang et al., 2014). In heparin, sulfate moieties render this oligosaccharide heavily negatively charged (Rabenstein, 2002). It is worth noting that many known heparin binding motifs exhibit multiple, evenly spaced basic residues (Capila and Linhardt, 2002). Overall, we thus suggest that electrostatic interactions between the positively charged YadA\textsubscript{O,9} loop residues and negatively charged functional groups on glycans are key to the binding affinity between YadA\textsubscript{O,9} and glycans.

In terms of biological relevance, we hypothesize the interaction with YadA\textsubscript{O,9} to be an additional mechanism for binding to host cell surfaces. As many secreted eukaryotic proteins are glycosylated for protein stability in the extracellular space (Varki et al., 2009), glycosylated ECM proteins could make a prime adhesion target during infection. Furthermore, a major group of molecules found in the ECM are GAGs such as heparin and heparan sulfate (Frantz et al., 2010). In addition to glycan adhesion being beneficial for the pathogen, it can conceptually be used in diagnostic workflows, e.g., to enrich pathogens from biological samples and potentially to develop anti-infective drugs. As glycans play a crucial role in pathogen adhesion, they have been used previously as therapeutics. Examples are the use of D-mannose in the treatment of urinary tract infections or of glycan derivatives to treat influenza (Domenici et al., 2016; Rustmeier et al., 2019; Weiss et al., 2020).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

IM: data acquisition, data visualization, methodology, writing of the original draft, and project conceptualization. JL-B: data acquisition, methodology, data visualization, and draft writing and review. PM, MS, and SP: conceptualization, and draft review and editing. DL: project administration, project conceptualization, and draft writing and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Horizon 2020 Innovative Training Network “ViBrANT” (to DL, SP, and PM) (funding ID: 765042). Contributions by the University of Oslo are gratefully acknowledged.

ACKNOWLEDGMENTS

We thank D. Hatlem and Ana Lucía Campaña (University of Oslo) for helpful discussions and T. Spåth (Institute for Medical Microbiology, Tubingen) for technical assistance. We thank Kristian Prydz for helpful discussions concerning this manuscript. We furthermore thank the imaging platform (NorMIC), especially Frode Miltzow Skjeldal, for help with the image analysis and quantification.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.738818/full#supplementary-material
REFERENCES

Ahmed, A., Rushworth, J. V., Wright, J. D., and Millner, P. A. (2013). Novel impedimetric immunosensor for detection of pathogenic bacteria Streptococcus pyogenes in human saliva. AnaL. Chem. 85, 12118–12125. doi: 10.1021/ ac403253f

Capila, I., and Linhardt, R. J. (2002). Heparin-protein-wechselwirkungen. Angew. Chem. 114, 426–450. doi: 10.1002/1521-3757(20020201)114:3<426::aid-ange26>3.0.co;2-q

Chauhan, N., Hatlem, D., Orwrick-Rydmark, M., Schneider, K., Floetenmeyer, M., van Rossum, B., and et al. (2019). Insights into the autotransport process of a trimeric autotransporter, Yersinia Adhesin A (YadA). Mol. Microbiol. 111, 844–862. doi: 10.1111/mmi.14195

Chauhan, N., Wrobel, A., Skurnik, M., and Leo, J. C. (2016). Yersinia adhesins: an arsenal for infection. Proteomics Clin. Appl. 10, 949–963. doi: 10.1002/prca.201600102

Domenici, L., Monti, M., Bracchi, C., Giorgini, M., Colagiovanni, V., Muzzi, L., et al. (2016). D-mannose: a promising support for acute urinary tract infections in women. A pilot study. Eur. Rev. Med. Pharmacol. Sci. 20, 2920–2925.

Frantz, C., Stewart, K. M., and Weaver, V. M. (2010). The extracellular matrix at a glance. J. Cell Sci. 123, 4195–4200. doi: 10.1242/jcs.023820

Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A. III, and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–345. doi: 10.1038/nmeth.1318

Grosskinsky, U., Schütz, M., Fritz, M., Schmid, Y., Lamparter, M. C., Szczesny, P., et al. (2007). A conserved glycine residue of trimeric autotransporter domains plays a key role in Yersinia adhesin a autotransport. J. Bacteriol. 189, 9011–9019. doi: 10.1128/jb.00985-07

Guán, J., Bywaters, S. M., Brendle, S. A., Ashley, R. E., Makov, A. M., Conway, J. F., et al. (2017). Cryo-electron microscopy maps of human papillomavirus 16 reveal L2 densities and heparin binding site. Structure 25, 253–263. doi: 10.1016/j.str.2016.12.001

Hayashi, M., Akama, T., Kono, I., and Kashiwagi, H. (1985). Activation of vitronectin (serum spreading factor) binding of heparin by denaturing agents. J. Biochem. 98, 1135–1138. doi: 10.1093/oxfordjournals.jbchem.a135363

Hwang, H., Lee, J. Y., Lee, H. K., Park, G. W., Jeong, H. K., Moon, M. H., et al. (2014). In-depth analysis of site-specific n-glycosylation in vitronectin from human plasma by tandem mass spectrometry with immunoprecipitation. Anal. Bioanal. Chem. 406, 7999–8011. doi: 10.1007/s00214-016-2822-6

Izumi, M., Yamada, K. M., and Hayashi, M. (1989). Vitronecin exists in two structurally and functionally distinct forms in human plasma. Biochim. Biophys. Acta Gen. Subj. 990, 101–108. doi: 10.1016/S0304-4165(89)80019-4

Keller, B., Mühlenkamp, M., Deuschle, E., Siegfried, A., Mössner, S., Schade, J., et al. (2015). Yersinia enterocolitica exploits different pathways to accomplish adhesive and toxin injection into host cells. Cell. Microbiol. 17, 1179–1204. doi: 10.1111/cmi.12429

Khow, O., and Suntrarachun, S. (2012). Strategies for production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. Mol. Cell. Biochem. 307, 249–264. doi: 10.1007/s11010-007-9603-6

Nesterenko, M. V., Titley, M., and Upton, S. J. (1994). A simple modification of blum’s silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. J. Biochem. Biophys. Methods 28, 239–242. doi: 10.1016/0165-1223(94)90020-5

Nguyen, T. Q., and Breitkopf, C. (2018). Determination of diffusion coefficients using impedance spectroscopy data. J. Electrochem. Soc. 165, E826–E831. doi: 10.1149/2.1151814jes

Pepe, J. C., Wachtel, M. R., Wagner, E., and Miller, V. L. (1995). Pathogenesis of defined invasion mutants of Yersinia enterocolitica in a BALB/c mouse model of infection. Infect. Immun. 63, 4837–4848. doi: 10.1128/iai.63.12.4837-4848.1995

Rabenstein, D. L. (2002). Heparin and heparan sulfate: structure and function. Nat. Prod. Rep. 19, 312–331. doi: 10.1039/s0109116

Rajas, O., Quirós, L. M., Ortega, M., Vazquez-Espinosa, E., Merayo-Lloves, J., Vazquez, F., et al. (2017). Glycosaminoglycans are involved in bacterial adherence to lung cells. BMC Infect. Dis. 17:319. doi: 10.1186/s12879-017-2418-5

Rustmeier, N. H., Strebl, M., and Stehle, T. (2019). The symmetry of viral sialic acid binding sites-implications for antiviral strategies. Viruses 11, 1–15. doi: 10.3390/v11090947

Saavedra, S., Kathatt, S. K., and Saini, K. S. (2008). Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. Mol. Cell. Biochem. 307, 249–264. doi: 10.1007/s11010-007-9603-6

Saragliadis, A., and Linhardt, R. J. (2019). Assay development for the discovery of small-molecule inhibitors of YadA adhesion to collagen. Cell Surface 5:100025. doi: 10.1186/s12879-017-00025

Sauer, M. M., Jakob, R. P., Lubke, T., Canonicum, F., Navarra, G., Ernst, B., et al. (2019). Binding of the bacterial Adhesin FimH to its natural, multivalent high-mannose type glyccan targets. J. Am. Chem. Soc. 141, 936–944. doi: 10.1021/jacs.8b10736

Schindler, M. K. H., Schütz, M. S., Mühlenkamp, M. C., Roosijakkers, S. H. M., Hallstrom, T., Zipfel, P. F., et al. (2012). Yersinia enterocolitica YadA mediates complement evasion by recruitment and inactivation of C3 products. J. Immunol. 189, 4900–4908. doi: 10.4049/jimmunol.1201383

Schulze-Koops, H., Burkhardt, H., Heesemann, J., Kirsch, T., Swoboda, B., Bull, C., et al. (1993). Outer membrane protein YadA of enteropathogenic yersiniae mediates specific binding to cellular but not plasma fibronectin. Infect. Immun. 61, 2513–2519. doi: 10.1128/iai.61.7.2513-2519.1993

Shahid, S. A., Nagaraj, M., Chauhan, N., Franks, T. W., Bardiaux, B., Habeck, M., et al. (2015). Solid-state NMR study of the YadA Membrane-anchor domain in the bacterial outer membrane. Angew. Chem. Int. Edn. 54, 12602–12606. doi: 10.1002/anie.201505006

Shriver, Z., Capila, I., Venkataraman, G., and Sasisekharan, R. (2012). Heparin and heparan sulfate: analyzing structure and microheterogeneity. Handb. Exp. Pharmacol. 207, 159–176. doi: 10.1007/978-3-642-23056-1_3
Meuskens et al. YadA Binds to Glycans

Sorin, M. N., Kuhn, J., Stasiak, A. C., and Stehle, T. (2021). Structural insight into non-enveloped virus binding to glycosaminoglycan receptors: a review. Viruses 13, 1–11. doi: 10.3390/v13050800

Stockmann, A., Hess, S., Declerck, P., Timpl, R., and Preissner, K. T. (1993). Multimeric vitronectin. Identification and characterization of conformation-dependent self-association of the adhesive protein. J. Biol. Chem. 268, 22874–22882. doi: 10.1016/s0021-9258(18)41608-0

Tamm, A., Tarkkanen, A. M., Korhonen, T. K., Kuusela, P., Toivanen, P., and Skurnik, M. (1993). Hydrophobic domains affect the collagen-binding specificity and surface polymerization as well as the virulence potential of the YadA Protein of Yersinia enterocolitica. Mol. Microbiol. 10, 995–1011. doi: 10.1111/j.1365-2958.1993.tb00971.x

Terti, R., Skurnik, M., Vartio, T., and Kuusela, P. (1992). Adhesion protein YadA of Yersinia species mediates binding of bacteria to fibronectin. Infect. Immun. 60, 3021–3024. doi: 10.1128/iai.60.7.3021-3024. 1992

Toivanen, P., and Skurnik, M. (1992). LcrF is the temperature-regulated activator of the YadA gene of Yersinia enterocolitica and Yersinia pseudotuberculosis. J. Bacteriol. 174, 2047–2051. doi: 10.1128/jb.174.6.2047-2051.1992

Trunk, T., Khalil, H. S., and Leo, J. C. (2018). Bacterial autoaggregation. AIMS Microbiol. 4, 140–164. doi: 10.3934/microbiol.2018.1.140

Varki, A., Esko, J. D., and Colley, K. J. (2009). Essentials of Glycobiology, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Vizarraga, D., Torres-Puig, S., Aparicio, D., and Pich, O. Q. (2021). The sialoglycan binding adhesins of Mycoplasma genitalium and Mycoplasma pneumoniae. Trend Microbiol. 29, 477–481. doi: 10.1016/j.tim.2021.01.011

Weiss, G. L., Stanisich, J. J., Sauer, M. M., Lin, C.-W., Eras, J., Zyla, D. S., et al. (2020). Filaments in urinary tract infections. Science 369, 1005–1010. doi: 10.1126/science.aaz9866

Westerlund, B., and Korhonen, T. K. (1993). Bacterial proteins binding to the mammalian extracellular matrix. Mol. Microbiol. 9, 687–694. doi: 10.1111/j.1365-2958.1993.tb01729.x

Zhuang, P., Li, H., Williams, I. G., Wagner, N. V., Seiffert, D., and Peterson, C. B. (1996). Characterization of the denaturation and renaturation of human plasma vitronectin II. Investigation into the mechanism of formation of multimers. J. Biol. Chem. 271, 14333–14343. doi: 10.1074/jbc.271.24.14333

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Meuskens, Leva-Bueno, Millner, Schütz, Peyman and Linke. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.