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# Assessment of IgG-Fc glycosylation from individual RhD-specific B cell clones reveals regulation at clonal rather than clonotypic level

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## Abstract

The type and strength of effector functions mediated by immunoglobulin G (IgG) antibodies rely on the subclass and the composition of the N297 glycan. Glycosylation analysis of both bulk and antigen-specific human IgG has revealed a marked diversity of the glycosylation signatures, including highly dynamic patterns as well as long-term stability of profiles, yet information on how individual B cell clones would contribute to this diversity has hitherto been lacking. Here, we assessed whether clonally related B cells share N297 glycosylation patterns of their secreted IgG. We differentiated single antigen-specific peripheral $IgG^+$ memory B cells into antibody-secreting cells and analysed Fc glycosylation of secreted IgG. Furthermore, we sequenced the variable region of their heavy chain, which allowed the grouping of the clones into clonotypes. We found highly diverse glycosylation patterns of culture-derived IgG, which, to some degree, mimicked the glycosylation of plasma IgG. Each B cell clone secreted IgG with a mixture of different Fc glycosylation patterns. The majority of clones produced fully fucosylated IgG. B cells producing afucosylated IgG were scattered across different clonotypes. In contrast, the remaining glycosylation traits were, in general, more uniform. These results indicate IgG-Fc fucosylation to be regulated at the single-clone level, whereas the regulation of other glycosylation traits most likely occurs at a

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clonotypic or systemic level. The discrepancies between plasma IgG and culture-derived IgG, could be caused by the origin of the B cells analysed, clonal dominance or factors from the culture system, which need to be addressed in future studies.

**KEYWORDS**
antibodies, B cell, B cell receptor, cell differentiation, repertoire development

## INTRODUCTION

Antibodies form a vital adaptive layer of humoral immunity and are produced by B cells, terminally differentiated into antibody-secreting cells (ASC). The binding of antigen enables antibody oligomerisation or immune complex formation, which facilitates binding by effector molecules such as C1q for complement activation or Fc receptors on myeloid cells and NK cells, potentially leading to antibody-dependent cellular phagocytosis or antibody-dependent cellular cytotoxicity (ADCC), respectively [1].

Although generally providing protection against pathogens, antibodies can elicit undesirable effects in autoimmune and alloimmune responses [2]. Furthermore, exploitation of antibodies by pathogens or excessive immune activation caused by triggering of either Fc receptors or complement during infections can be deleterious to the host [3–7]. Immunoglobulin G (IgG) is the most abundant antibody isotype in plasma and mediates protection through all the effector mechanisms described above. However, the strength and type of effector function are governed by the affinity to effector molecules and overall avidity in the immunological synapses, which differs between subclasses (IgG1–4) [8]. Furthermore, the specific glycan structure at the highly conserved site in the CH2 domain of the Fc region modulates the affinity to effector molecules. This *N*-linked glycan at position N297 is composed of a diantennary core structure of four *N*-acetylglucosamines (GlcNAc) and three mannoses, which can be further modified with a core fucose and/or a bisecting GlcNAc [9]. Moreover, each antenna can be elongated with galactose and sialic acid [9]. While increased galactosylation and sialylation enhance IgG-Fc multimerisation and the complement-activating capacity of IgG [10, 11], only core fucosylation influences the IgG's affinity to IgG-Fc receptors (FcγR) significantly. IgG lacking core fucose (afucosylated) has up to 40-fold increased affinity to FcγRIII [12, 13] and as a consequence of this, afucosylated IgG can induce strong FcγRIII-dependent effector functions such as NK cell-mediated ADCC. This response is often amplified well beyond 40-fold due to the increased avidity between target and FcγRIII-expressing cells [14]. Afucosylated IgG is rare in plasma (∼6%), but interestingly, the composition of the N297 glycan depends on the antigen-specificity. For instance, afucosylated IgG is enriched in responses against the alloantigens RhD [15] and human platelet antigen-1a (HPA-1a) [16, 17], as well as in responses towards surface antigens of enveloped viruses [6, 18, 19] and *Plasmodium falciparum* [20]. Fc fucosylation of RhD and HPA-1a alloantibodies has proved the best variable for predicting disease severity in sensitised pregnancies, highlighting the importance of preventing the formation of afucosylated IgG in these settings.

Whereas Fc galactosylation and sialylation levels of both total IgG and antigen-specific IgG are rather dynamic, Fc fucosylation exhibits long-term stability, suggesting different regulation of the glycosylation traits [16, 21–23]. Whilst there is a large body of literature on plasma IgG glycosylation, the regulation of Fc glycosylation in B cells is still largely unexplored.

Here, we used an in vitro culture system to differentiate single primary RhD-specific memory B cells into ASC, allowing us to analyse the IgG glycosylation on a clonal level. We defined a clone as a B cell with a unique heavy chain variable region and compared that to the glycosylation of RhD-specific IgG isolated from plasma of the same individual. We furthermore grouped B cell clones with identical V-D-J rearrangement into clonotypes and resolved their intra-clonotypic relationship based on somatic hypermutations. This provided new insights into the diversity and conservation of Fc glycosylation at both clonal and clonotypic levels.

## MATERIALS AND METHODS

### Ethical approval

EDTA blood samples were collected from RhD hyperimmunised donors by Sanquin Blood Supply (Amsterdam, The Netherlands). All donors completed an informed consent, and the study was approved by the ethics advisory board of our institute.

## RhD-specific IgG isolation from human plasma samples

RhD-specific antibodies were captured on washed $RhD^+$ red blood cells (packed RBC; Genotype R2R2). EDTA plasma from hyperimmune donors was stored at −20°C until antibody purification. Plasma samples were incubated with packed RBC (1 h; 37°C; 1–0.25 mL plasma depending on titre). Cells were spun down (1 min; 2300 × *g*), and the remaining plasma was aspirated, followed by six-time washes with 10 mL phosphate-buffered saline (PBS; 4°C). Antibodies were eluted with PeliStrip (500 µL; Sanquin, Amsterdam, The Netherlands), after which the RBC were spun down (2.5 min; 1200 × *g*) and the supernatants were transferred to tubes containing neutralising buffer (Tris [214 mM], $Na_2HPO_4$ [22 mM], pH 10). pH was checked and adjusted to 7–8. Leftover RBC debris in the eluates was pelleted (1 min; 20 000 × *g*) and supernatants were transferred to fresh low-binding tubes. IgGs were further purified from eluates by applying the eluates to protein G-coupled cartridges of the AssayMAP Bravo, as described below.

## Purification of IgG from human plasma samples

Total plasma IgG was purified using the AssayMAP Bravo (Agilent Technologies, Santa Clara, USA) with protein G-coupled cartridges. Plasma samples were diluted in PBS (1:100 in 100 µL) and applied to the cartridges, followed by washes of PBS, UPLC/MS grade water (Biosolve, Dieuze, France;10 column volumes of each) and finally eluted with formic acid (1%).

## Preparation of RhD red cell ghosts

$200 \times 10^6$ untreated $RhD^+$ RBC (Genotype R2R2) were washed three times with ice-cold $NaH_2PO_4$ (5 mM; pH 8.2) supplemented with protease inhibitor ($NaH_2PO_4$-PI; 20 000 × *g*; 4°C; 20 min). Ghosts were then stained with CellTrace™ Violet (20 µM), CellTrace™ FarRed (10 µM), or CellTrace™ CFSE (20 µM), or PKH26 Red Fluorescent Cell Linker (2 µM) in $NaH_2PO_4$-PI (15 min; 37°C). The stains were subsequently quenched by washing three times with $NaH_2PO_4$–PI supplemented with human serum albumin (1%; 30 min; 20 000 × *g*; 4°C) followed by incubation in the same buffer (30 min; 37°C). Ghosts were finally washed two times with $NaH_2PO_4$–PI and stored in Iscove's modified Dulbecco's medium (IMDM) supplemented with fetal calf serum (FCS; 10%), penicillin (100 units/mL), streptavidin (100 µg/mL) and L-glutamine (2 mM).

## Sorting and culturing of single B cell

B cells were isolated from peripheral blood from hyperimmunised donors at varies times after immunisation. Briefly, whole blood was diluted with an equal amount of PBS fractionated by Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare, Chicago, USA; 420 g; 30 min; no brake) and the cellular ring containing the peripheral mononuclear blood cells (PBMC) was isolated and washed two times with PBS. B cells were enriched from PBMC by CD19 Pan B Dynabeads (20 min; 4°C; while rotating; Invitrogen, Carlsbad, USA) in PBS supplemented with 0.1% bovine serum albumin (PBS + 0.1% BSA). Beads and captured cells were washed three times with PBS + 0.1% BSA, followed by incubation with DETACHaBEAD CD19 (Invitrogen, Carlsbad, USA; 45 min; room temperature; while rotating). Released cells were collected and washed three times with PBS. Cells were spun down, and stained in IMDM supplemented with IgG-depleted FCS (5%), human IgG-free apotransferrin (20 µg/mL; Sigma-Aldrich) and 2.5% T cell supernatant (derived from human T cells stimulated for 36 h with PHA-L L [Murex, Dartford, UK] and PMA [Sigma-Aldrich]), R848 (0.5 µg/mL), penicillin (100 unit/mL) and streptavidin (100 µg/mL) and L-glutamine (2 mM) (dFCS medium). B cells were stained (25 min; 4°C) with fluorescein isothiocyanate-conjugated anti-human CD19 (CLB-B4/1 1:200; Sanquin, Amsterdam, The Netherlands), and phycoerythrin/Cyanine7-conjugated mouse anti-human CD27 (Clone M-T271; 1:20, BD, Franklin Lakes, USA). Following one wash with dFCS medium, the cells were co-incubated with stained bromelain-treated R2R2 ghosts of two colours (1:6 ratio B cell/ghost; 45 min; 4°C; while rotating). The cells were washed once with dFCS medium, after which cells were suspended in dFCS medium and sorted using a BD FACSAria II. Single $CD19^+$ $ghost^{++}$ cells were sorted into 96-well plates prepared with 100 000 irradiated EL4.B5 cells (50 grey) per well in dFCS medium as previously described [24]. Only B cells-binding RBC ghosts of both fluorescent dyes were considered RhD-specific to eliminate specific binders. Cells were cultured for 7–16 days (37°C; 5% $CO_2$), after which supernatants were harvested and tested for antibody content (IgG ELISA) and specificity (micro agglutination assay) (see below). Cells from $antibody^+$ wells were lysed in RLT plus buffer (Qiagen, Hilden, Germany) for later RNA purification. IgG from cell supernatant was

harvested using protein G-coupled cartridges on the AssayMAP Bravo as described above.

## IgG ELISA

Supernatants from B cell cultures were checked for the presence of IgG. Flat-bottomed 96-well microtiter plates (Maxisorp, Nunc Roskilde, Denmark) were coated with mouse anti-human IgG (Clone JDC-10; 0.66 μg/mL; Southern Biotech; Birmingham; USA) in PBS (overnight; 4°C). Wells were blocked with PBS-T with 2% skim milk powder (Campina, The Netherlands; 1 h; room temperature), followed by incubation with the cell supernatants diluted 1:2 in PBS (1 h; room temperature). Plates were then incubated with biotinylated anti-IgG (Clone JDC-10; 0.1 μg/mL; Southern Biotech; Birmingham, AL, USA) (1 h; room temperature) in PBS-T, followed by incubation with poly horseradish peroxidase-conjugated streptavidin (0.1 μg/mL; Sanquin; Amsterdam; The Netherlands) in PBS-T (30 min; room temperature). Plates were developed with TMB substrate (sodium acetate 0.1 M, pH 5.5; 3,3′,5,5′-tetramethylbenzidine 0.1 mg/mL; $H_2O_2$ 0.03%), terminated with $H_2SO_4$ (2 M), and optical densities were read at 450 nm with background at 540 nm deducted. Plates were washed with PBS-T five times between all incubations.

## Micro agglutination assay

Agglutination assays were performed in U-bottomed plates (Greiner) with $RhD^+$ (R2R2) and $RhD^-$ (rr) erythrocytes. $0.5–1 \times 10^6$ bromelain-treated $RhD^-$ erythrocytes were incubated with 25 μL of IgG-positive B cell supernatant (20 min; 37°C). Cells were afterwards spun down (400 × *g*; 2 min) and supernatants were transferred to plates with bromelain-treated $RhD^+$ erythrocytes followed by an additional wash with PBS + 0.1% BSA (400 × *g*; 2 min). Twenty microlitres of Coombs serum was incubated with the erythrocytes (10 min; room temperature), after which the cells were spun down (400 × *g*; 1 min) and agglutination was manually inspected.

## Mass spectrometric IgG-Fc glycosylation analysis

Eluates containing IgG were collected using the AssayMAP Bravo as described above in low-protein-binding PCR plates (Eppendorf, Hamburg, Germany) and dried by vacuum centrifugation (50°C). Dried samples were dissolved in a reduction and alkylation buffer containing sodium deoxycholate (SDC; 0.4%), tris(2-carboxyethyl) phosphine (10 mM), 2-chloroacetamide (40 mM), TRIS (pH 8.5; 100 mM). After 10 min at 95°C, the samples were enzymatically digested by trypsin (5 μg/mL) in ammonium bicarbonate (50 mM). The digestion was terminated after overnight incubation (37°C) by acidifying to 2% formic acid. Before mass spectrometry injection, SDC precipitates were removed by centrifuging samples (3000 × *g*; 30 min), and filtering through 0.65 μm low-protein-binding filter plates (Millipore, Burlington, USA). Analysis of IgG-Fc glycosylation was performed using reversed-phase nanoLC coupled to an Impact HD or maXis quadrupole time-of-flight-MS (q-TOF-MS; Bruker Daltonics, Bremen, Germany) equipped with a nano-Booster (Bruker Daltonics). Analysis performed on Impact HD is described elsewhere [25]. The nanoLC set-up was equipped with an Acclaim PepMap 100 trap column (100 μm × 20 mm, particle size 5 μm; Thermo Fisher Scientific, Waltham, MA) and an Acclaim PepMap RSLC C18 nano-column (75 μm × 150 mm, particle size 2 μm; Thermo Fisher Scientific). The sample was injected and the (glyco)peptides were separated with a flow rate of 700 nL/min and a linear gradient from 97% Solvent A (0.1% formic acid in water) and 3% Solvent B (95% ACN) to 27% Solvent B over 15 min. The following MS settings were applied: mass range $m/z$ 550–1800; spectral acquisition frequency 1 Hz; collision energy 5.0 eV, transfer time 130 μs, pre-pulse storage 10 μs. Mass spectrometry results were analysed with Skyline software [26]. Glycoforms were manually assessed for each clone and included in the analysis if a clear peak with expected retention time and isotopic distribution was identified. The level of fucosylation and bisection were calculated as the summed relative intensities of glycoforms containing the respective glycotraits. Levels of galactosylation and sialylation were calculated as antenna occupancy, where the relative intensities of the glycoforms were summed with mono-galactosylated/sialylated species only contributing 50% of their relative intensity.

## Sequencing of IGHV region

The protocol was adapted from elsewhere [27]. Stored RNA was purified from RTL plus (Qiagen, Hilden, Germany) using RNeasy Plus Micro Kit (Qiagen) according to manufacture's protocol. cDNA was synthesised using SuperScript III First-strand System (Thermo Fisher Scientific) and random hexamers. Reactions were brought to 50°C for 50 min, 4°C for 2 min and 25°C for 10 min. cDNA was synthesised using the manufacture-specified mastermix and was done at 50°C for 50 min,

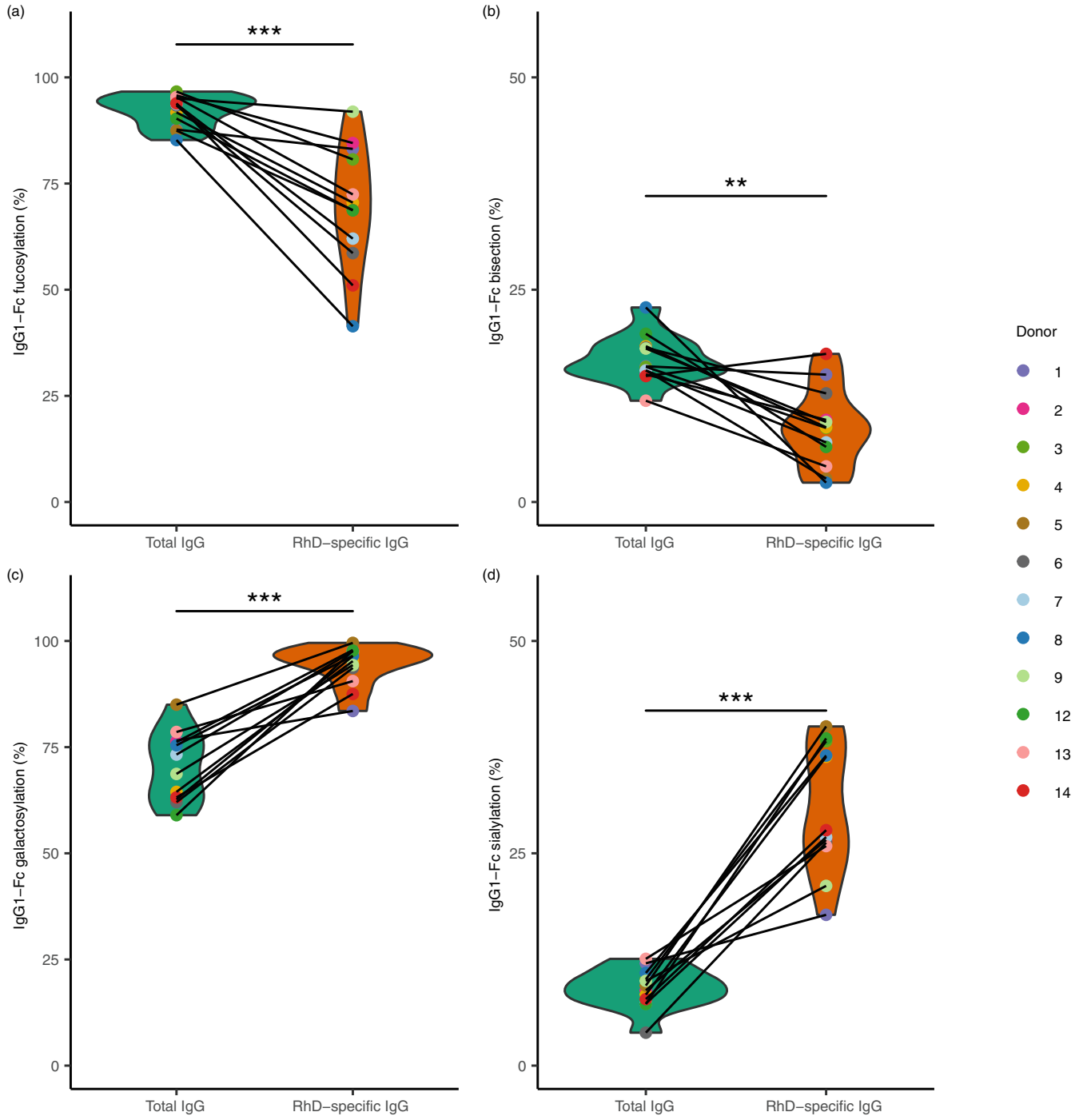


**FIGURE 1** Fc glycosylation of RhD-specific plasma IgG1 is significantly different from total plasma IgG. IgG-Fc glycosylation of plasma-derived RhD-specific IgG1 and total plasma IgG1 is shown. (a) Fc fucosylation, (b) Fc bisection, (c) Fc galactosylation and (d) Fc sialylation were compared between antigen-specific and total IgG. Statistically significant pairwise differences between antigen-specific IgG and total IgG (two-sided Wilcoxon signed rank tests) are indicated (**$p < 0.01$; ***$p < 0.001$).

followed by 5 min at 85°C. Finally, all wells were RNase H treated for 20 min at 37°C.

The IgG heavy chain variable regions were amplified from the cDNA using AmpliTaq Gold DNA polymerase (0.5 U/reaction) in Gold buffer II supplemented with MgCl (2.5 mM), dNTP (0.2 mM of each) and IGH-VJ primer pool (2.3 mM). PCR conditions were: 10 min at 94°C, 35 × (1 min at 94°C, 1 min at 63°C and 30 s at 72°C), followed by a final elongation step of 5 min at 72°C.

Products were then coupled to Ion Torrent specific adaptors (P1-, A- adaptors and barcodes) for

next-generation sequencing (NGS), during a second PCR, using Fast Start HiFi Taq (2.5 U/reaction) in FS HiFi buffer supplemented with MgCl (1.8 mM), dNTP (0.2 mM of each), trP1MKIE primer and AMIDxx primer (0.2 mM). PCR conditions were: 2 min at 94°C, 20 × (30 s at 94°C, 30 s at 63°C and 30 s at 72°C), followed by a final elongation step of 5 min at 72°C. The final PCR products were run in an agarose gel to verify product size and purity. In samples with a high amount of primer dimers, the specific band was excised from the agarose gel or otherwise cleaned up using ExoSap (Applied Biosystems). NGS was done using the IonTorrent S5 system, and unprocessed sequencing reads (fastq) were analysed by Arrest/Interrogate software [28].

## Construction of clonal trees

B cell clones were grouped into clonotypes based on identical VDJ usage and length of the CDR3. The relationship between each clone in the clonotypes were determined manually, by aligning the sequences of the germline IGVH gene, and looking for shared base pair mutations, and further validated using the dowser package (version 1.0.0) [29] using maximum parsimony, where similar annotation of V and J genes and a CDR3 similarity of 85% nt level (hamming distance) was used as a threshold for clonal grouping.

# RESULTS

## RhD-specific plasma IgG has altered IgG glycosylation profiles compared to total plasma IgG

We first assessed the Fc glycosylation of plasma-derived IgG from 12 hyperimmunised RhD donors and compared the Fc glycosylation of the total plasma IgG to that of RhD-specific IgG using LC-MS. Reaffirming previous results [15, 30], decreased levels of Fc fucosylation of the RhD-specific IgG1 compared to the total plasma IgG1 were observed (Figure 1a). Similarly, bisection of RhD-specific IgG1 (Figure 1b) was decreased compared to total plasma IgG1, whereas galactosylation (Figure 1c) and sialylation (Figure 1d) was increased compared to total plasma IgG1. As the glycopeptides of IgG2 and IgG3 have identical masses and retention times, we were unable to distinguish IgG2 and IgG3 in our analysis. However, RhD-specific alloantibodies have invariably been shown to be of IgG1 and IgG3 subclass [31–33]. Differences between glycosylation of total and antigen-specific IgG2/3 antibodies were similar to the differences of IgG1, except for the levels of bisecting GlcNAc (Figure S1). Collectively, RhD-specific IgG from the hyperimmunised donors exhibited distinctive glycosylation profiles different from total plasma IgG.

## Single RhD-specific B cells produce IgG with highly specific Fc fucosylation profiles

We next sought to characterise antigen-specific IgG-Fc glycosylation on a clonal B cell level. To achieve this, single $CD19^{+}$ RhD-specific lymphocytes isolated from peripheral blood were co-cultured with irradiated CD40L-expressing EL4.B5 cells. Likewise, B cells not specific for RhD were cultured as controls. Following culture and confirmation of IgG production and RhD-specificity, IgG was purified from culture supernatants and Fc glycosylation was assessed by LC-MS (Figure 2a). We successfully isolated and glyco-profiled culture-derived IgG from six donors. A variety of glycoforms was identified for IgG derived from single B cell clones, similar to plasma IgG, despite being monoclonal (Figure S1). The average of glycotraits of IgG from different cultured clones mirrored their respective plasma counterparts well within donors (Figures 2b–e and S2). The exceptions to this were higher degrees of bisection and lower degrees of galactosylation of RhD-specific IgG, compared to the RhD-specific IgG from plasma (Figures 2b–e and S2). Of note, both RhD-specific and non-RhD-specific clones displayed bisection, galactosylation, and sialylation degrees in narrow and uniform ranges within donors. In contrast, only non-RhD-specific clones displayed a narrow range of fucosylation, which was almost exclusively >95% (Figures 2b–e and S2). RhD-specific clones producing partly afucosylated IgG1 and/or IgG2/3 were identified across all donors (fucosylation degrees of 25%–90%; Figures 2b and S2). Despite the identification of clones producing afucosylated IgG, the majority of the RhD-specific clones produced fully fucosylated IgG, higher than observed for the plasma-derived RhD-specific IgG (Figure 2b). Donors 3 and 13 were notable exceptions to this, as the majority of isolated B cell clones produced IgG with fucosylation degrees similar to both RhD-specific and total IgG from plasma in these donors (Figure 2b). Of note, a comparison of the glycosylation profiles across the culture-derived IgG revealed very similar glycosylation profiles in general and unveiled the biggest differences to exist between donors (Figure S3). Thus, IgG produced by cultured B cell clones exhibited a homogenous distribution of glycosylation profiles within donors. Furthermore, the

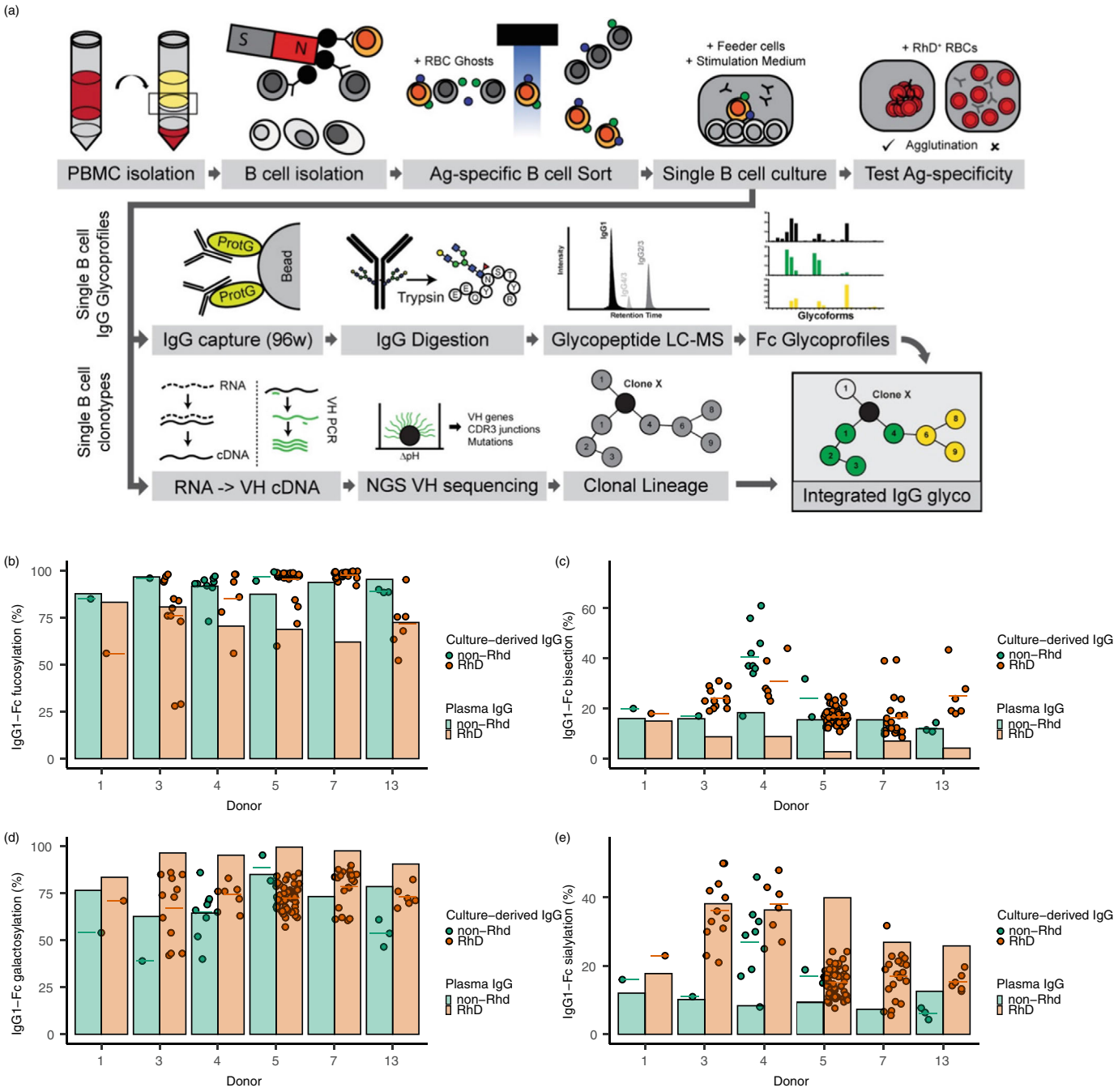


**FIGURE 2** Fc glycosylation of culture-derived IgG only partially mirror Fc glycosylation of plasma IgG. (a) Single memory B cells were differentiated into ASC allowing analysis of culture-derived IgG. Plasma IgG1 was compared to culture-derived IgG1 for both RhD-specific and total IgG in terms of (b) fucosylation, (c) bisection, (d) galactosylation and (e) sialylation. Bars and dots (b–e) represent IgG-Fc glycosylation traits of plasma- and culture-derived IgG1, respectively. Both non-RhD-specific (green) and RhD-specific (orange) IgG was assessed. Horizontal lines in matching colours represent the mean percentage of each glycosylation traits from culture-derived IgG.

mean of cultured-derived IgG had a high similarity to both the RhD-specific and total plasma IgG for most glycosylation traits. However, RhD-specific B cells exhibited bigger heterogeneity with regard to fucosylation, either producing fully fucosylated IgG or partly afucosylated IgG with widely varying fucosylation degrees.

## Fc glycosylation is regulated at a clonal level and not at a clonotypic level

To gain additional information about the regulation of IgG-Fc glycosylation, we resolved the clonal relationship of the analysed RhD-specific single B cell clones by sequencing the variable region of the heavy chain of the

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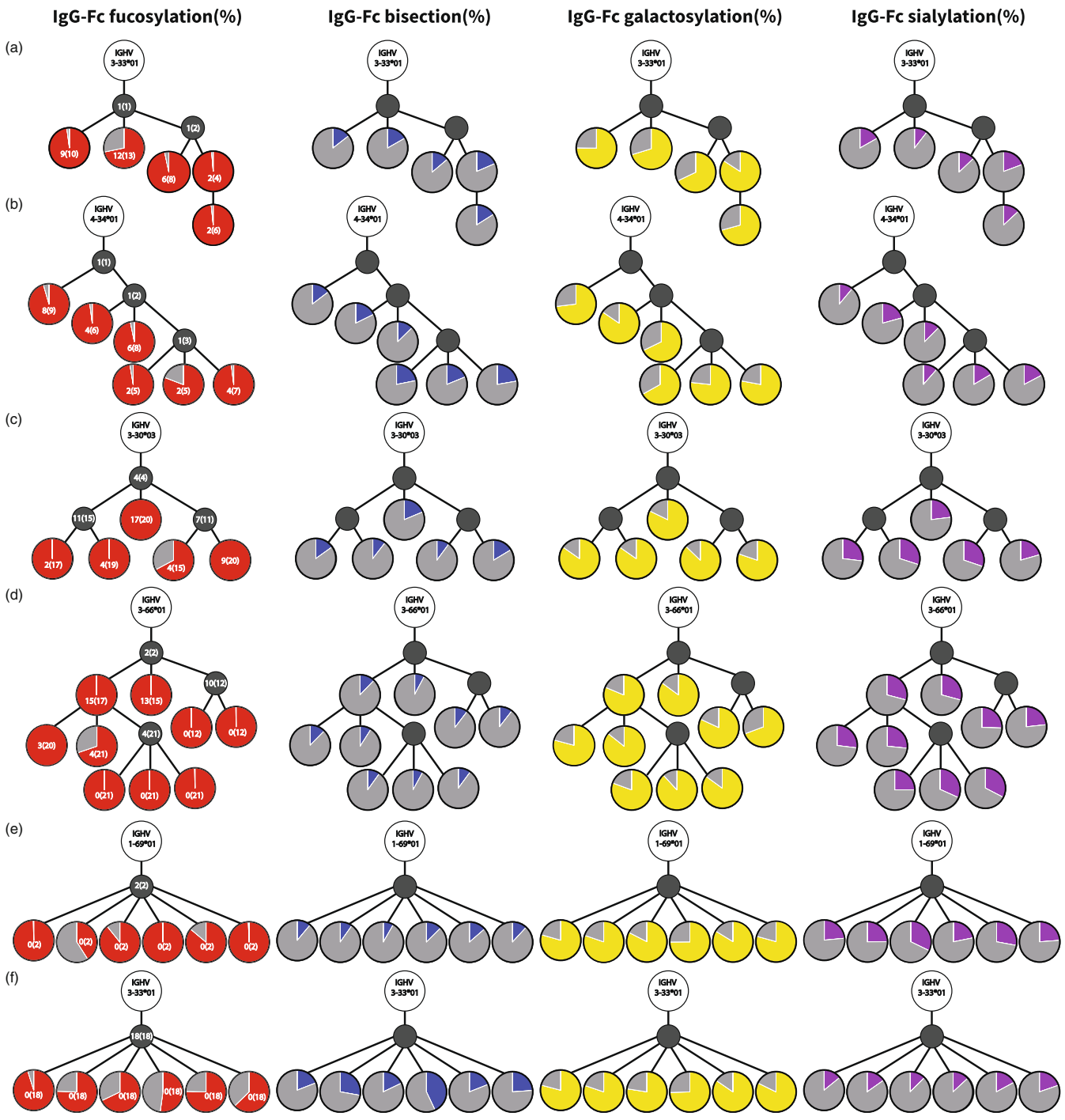


**FIGURE 3** Clonal trees of RhD-specific B cells display no clonotypic regulation of Fc glycosylation, but reveals a stochastic pattern of Fc glycosylation within clonotypes. ASC from (a, b) Donor 5, (c–e) Donor 7 and (f) Donor 13 were grouped into clonotypes based on their heavy chain variable region, and clonal trees were constructed based on accumulated mutations from the germline sequence. Each pie chart represents an analysed B cell clone. The number of mutations from previous clone is annotated unbracketed and the total number of mutations from the germline sequence is annotated in brackets in the leftmost trees. Each clonotype is depicted four times to show the percentage of each glycosylation trait from the respective clone as pie charts (red = fucosylation [leftmost trees], blue = bisection [centre left trees], yellow = galactosylation [centre right trees] and purple = sialylation [rightmost trees]). White notes represent the deduced germline clone and smaller dark grey notes represent inferred clones.

single clones (Figure 2a). Clones were assigned clonotypes based on VH- and J-gene usage, and CDR3 length and similarity. The intra-clonotypic relationships were determined by the number of accumulated mutations compared to the assigned germline VH-gene. The resulting clonal trees revealed a highly stochastic pattern of Fc

fucosylation, as clones producing afucosylated IgG belonged to clonotypes otherwise exclusively consisting of clones producing fully fucosylated IgG (Figures 3 and S4). In contrast, only small variations of the remaining IgG-Fc glycosylation traits were observed within clonotypes, but as the levels of these were more homogenous between clones, a clear pattern of clonotypic regulation was also absent for these (Figure 3). Two clonotypes (clonotypes IGHV1-69*05 and 3-33*01; Figure 3e,f, respectively), originating from two different donors, were of particular interest, as all analysed clones within each clonotype had identical variable region sequences, but varying Fc glycosylation profiles. One of these (clonotype IGHV3-33*01) showed multiple clones with low levels of Fc fucosylation. Interestingly, this clonotype was the only clonotype detected in Donor 13, which was the donor with the best association between plasma-derived and culture-derived RhD-specific IgG.

Thus, our results suggest no regulation of Fc fucosylation at a clonotypic level despite strongly skewed profiles of antigen-specific plasma IgG compared to total plasma IgG. Regulation of Fc fucosylation is, therefore, more likely regulated at the level of single B cell clones. In contrast, the more uniform distribution of the remaining glycosylation traits of the culture-derived IgG suggests these to be regulated at either systemic or clonotypic levels.

## DISCUSSION

We here analysed Fc glycosylation of RhD-specific IgG from in vitro cultured clonal B cells and matched plasma, and assessed possible regulation at a clonotypic level.

Despite the similarity of glycosylation traits of culture-derived IgG and the plasma-derived counterpart, we did observe origin-dependent differences. Although no statistically significant differences were found between plasma IgG and the mean of culture-derived IgG, Fc fucosylation of culture-derived IgG resonated poorly with the plasma-derived counterpart at the single clone level. This was exemplified by the fact that most clones producing fully fucosylated IgG were identified after culture. Plasma IgG is primarily produced by plasma cells in the bone marrow and not by peripheral B cells, as used in this study, possibly explaining this discrepancy. However, we did identify RhD-specific B cell clones producing afucosylated IgG and in two donors (Donors 3 and 13) we observed a high similarity of RhD-specific IgG-Fc fucosylation between the average of culture-derived IgG and plasma purified IgG. From Donor 13, we only identified clones with identical variable region sequences, suggesting this clonotype with one highly expanded clone to be highly dominant. If the dominance of this clone in peripheral blood extends to the bone marrow compartment, this could offer an explanation to the better correspondence between culture-derived and plasma IgG in this donor.

As multiple RhD-specific clones across donors produced afucosylated IgG in contrast to non-RhD clones, glycosylation traits appeared to be preserved in peripheral memory B cells and resulted in the production of afucosylated IgG once the B cell differentiated into an ASC. This imprinting is also in agreement with the fact that RhD-specific IgG-Fc fucosylation, and Fc fucosylation of IgG specific for HPA-1a and some viral antigens, are stable for at least a decade, even with multiple immunisations, in contrast to the other glycosylation traits [6, 16, 23, 30]. Our data are, however, only exploring RhD-specific responses and further analysis of more antigen-specific antibody responses is needed, to clarify if this is a general phenomenon. Whether the observed afucosylated IgG in some of the cultured B cell clones originate from a population of ASC, expanded from a single B cell with either homogeneous or heterogenous fucosylation patterns, is impossible to assess in the present study. However, either scenario most likely dictates IgG-Fc fucosylation to be a set trait by the mother B cell, resulting in either a population of ASC-secreting IgG with similar fucosylation degrees or in a heterogenous population with different fucosylation degrees. The observed differences in IgG-Fc fucosylation between different isolated B cell clones minimise the likelihood of culture conditions introducing strong biases of IgG-Fc fucosylation. This is in coherence with several studies which have shown cytokines to influence the galactosylation and sialylation, but not fucosylation of IgG in vitro [34, 35]. However, culture-dependent biases are impossible to exclude and should be considered for the analysis of our data.

The long-term sustained IgG-Fc fucosylation pattern of plasma IgG could imply fucosylation levels to be set in primary responses and remain stable in subsequent responses. The set glycosylation profiles might then be passed on to daughter cells as part of immunological memory by imprinting of memory B cells. However, we were unable to find signatures of such imprinting in the clonotypes analysed. Importantly, we were only able to construct rather small clonal trees of RhD-specific clones, which could limit our power to detect possible clonotypic regulation of IgG-Fc glycosylation. However, the data suggests that producers of afucosylated IgG may arise at least partly independently of mother-clones' fucosylation pattern, as discrepancies between mother and daughter clones were present in multiple clonotypes. Data from

antigen-specific plasmablasts and peripheral B cells from both SARS-CoV-2- and HIV-infected individuals suggest that the protein and mRNA levels, respectively, of FUT8, the glycosyltransferase responsible for core fucosylation in B cells, is decreased in individuals producing afucosylated IgG [19, 36, 37]. Furthermore, the transcription factors IKAROS and HNF1α have been associated with core fucosylation in genome-wide association studies, but the cellular mechanisms regulating afucosylated IgG remain unexplored [38, 39].

IgG-Fc galactosylation and sialylation levels of culture-derived IgG, were generally lower than the matched plasma IgG. As described above, this discrepancy could be caused by stimuli from the culture medium but also the timing of sampling, as the donors were recently boosted, which is known to increase Fc galactosylation and sialylation of plasma IgG [23]. This is most likely caused by the addition of newly formed plasma cells to the pool of antigen-specific B cells, which was unechoed by the cultured B cells in the present study, possibly due to the origin of the cells and lack of proper stimuli during culture. IgG-Fc bisection was generally higher for culture-derived IgG than for plasma IgG, mostly likely due to the confounders described above. All clones exhibited IgG-Fc galactosylation, sialylation and bisection levels in restricted ranges in stark contrast to the distribution of the fucosylation levels. As the Fc bisection, galactosylation, and sialylation all showed minor differences between clones, no clonal inheritance pattern of these glycosylation traits was evident. However, the low variance of these traits between clones could point to regulation at systemic or clonotypic level.

In conclusion, we here describe the, to our knowledge, first glycosylation analysis of IgG from single B cell clones in an antigen-specific system. This required highly sensitive assays, due to the low amount of IgG in the culture system, which extended the limit of available methods, and furthermore precluded analysis at earlier timepoints. We were unable to find any signs of clonotypic regulation of Fc glycosylation, as only single clones with unique glycosylation features were identified. Of these, fucosylation presented as the most extreme glycosylation trait by displaying a phenotype of either fully fucosylated IgG or, in a minority of clones, a wide range of afucosylation, which further supports the idea of afucosylated responses being a unique phenomenon differing from the other glycosylation traits of the IgG-Fc glycan in their regulation, magnitude and frequency. Despite being based on in vitro differentiated B cells, these data add important knowledge to the field of IgG-Fc glycosylation regulation and further highlight the importance of different B cell compartments and stimuli, which can result in IgG with highly different effector profiles due to Fc glycosylation. A rapidly expanding amount of data on plasma IgG-Fc glycosylation is being published, emphasising the importance of this post-translational modification of IgG in multiple settings, but the molecular mechanisms regulating this remain elusive. Future work will seek to pinpoint these and aim for thorough phenotyping of B cells capable of producing IgG with highly different glycosylation patterns.

## AUTHOR CONTRIBUTIONS

Erik L. de Graaf, Mads Delbo Larsen and Remco Visser cultured single B cells, purified RNA and performed PCR reactions. Erik L. de Graaf, Mads Delbo Larsen, Remco Visser, and Nieke van der Bolt purified IgG from plasma and clonal samples. Onno J. H. M. Verhagen performed the NGS of the variable region of the heavy chains. Agnes L. Hipgrave Ederveen and Carolien A. M. Koeleman performed glycosylation analysis. Mads Delbo Larsen and Erik L. de Graaf processed the glycosylation data. Mads Delbo Larsen and Nieke van der Bolt constructed clonal trees. The study was conceptualized by Erik L. de Graaf, Mads Delbo Larsen, C. Ellen van der Schoot, Manfred Wuhrer and Gestur Vidarsson. Initial manuscript was written by Mads Delbo Larsen and Gestur Vidarsson. All authors read and commented on the initial manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## INFORMED CONSENT

All donors completed an informed consent, and the study was approved by the ethics advisory board of our institute.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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