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Purification free N-glycan analysis by capillary zone electrophoresis: Hunt for the lost glycans

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A R T I C L E I N F O

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A B S T R A C T

Capillary gel electrophoresis is a widely used method for rapid separation of fluorophore labeled carbohydrates. Even though, many publications conferred about this popular technique, no report yet investigated the possible sample losses during the purification process of the fluorophore labeling reaction mixture. In the present work, normal polarity capillary zone electrophoresis separation mode was applied to take advantage of the opposite migration directions of the electroosmotic flow and the negatively charged sample components using Tris-hexanoic acid running buffer at basic pH. For purification free oligosaccharide analysis, the separation parameters were designed in such a way that the triple charged labeling reagent of aminopyrenetrisulfonate (APTS) could not enter the separation capillary in contrary to the labeled sample components of interest, therefore, the APTS did not have to be removed before analysis. The method was used to show electrophoretic profile differences possibly caused by the cleanup process that was immediately apparent by comparing the electropherograms of the purified and non-purified APTS labeled maltooligosaccharides. Furthermore, qualitative and quantitative N-glycosylation profile alterations were revealed during CZE separation of the fluorophore labeling reaction mixtures before and after purification along with the analysis of the consecutively used washing solutions for the well characterized standard glycoproteins of IgG, ribonuclease B and fetuin.

1. Introduction

Analysis of the carbohydrate moiety of glycoproteins is of growing interest, not only in the biopharmaceutical and clinical fields but also in the food-beverage sector [1,2]. Capillary electrophoresis (CE) employing a low pH gel-buffer system under arheic conditions is a commonly used separation method for glycan analysis [3]. Release of the asparagine linked oligosaccharides is most frequently mediated by endoglycosidases, mainly by peptide-N-glycanase F (PNGase F) [4]. Since carbohydrates lack UV- or fluorescently-active groups, their capillary electrophoresis analysis entails tagging of the free reductive end of the released sugars with a primary amine bearing fluorescent dye that is also charged to support proper electromigration [5]. The most commonly used derivatization agent in capillary electrophoresis is aminopyrenetrisulfonate (APTS) in a two-step reductive amination process based reaction [6]. The Schiff base, formed in the first step, is reduced in the second step to obtain a stable conjugate [7]. To assure high reaction yield, a great excess of the derivatization agent is used, orders of magnitude more than that of the equimolar ratio would entail. Therefore, a purification step is required in most instances before CE analysis of the labeled carbohydrates as the large peak of the non-reacted derivatization agent can overlap with some of the sample components of interest. Commonly used purification methods for capillary electrophoresis analysis of fluorophore labeled sugars include the use of hydrophilic interaction based microcolumns/plates [8] or carboxyl coated paramagnetic beads [9], both supporting full automation with the use of liquid handling robots. While these methods are reportedly capable to eliminate the majority of the excess derivatization agent along with most of the salts in the reaction mixture, at the best of our knowledge, no study was reported so far on the possible loss (qualitative or

Abbreviations: APTS, aminopyrenetrisulfonate; BFS, bare fused silica; BGE, background electrolyte; CGE, capillary gel electrophoresis; CZE, capillary zone electrophoresis; GU, glucose unit.

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quantitative) of some of the sample components during the washing steps of the cleanup processes.

In this work, we investigated the capillary electrophoresis based carbohydrate analysis workflow with a conceptually different separation mechanism of using basic pH conditions, which did not require pre-separation removal of the excess fluorophore after the labeling reaction. In this way, it was possible to comprehensively analyze the otherwise discarded high fluorophore content washing solutions during the sample purification steps to reveal the cause of any possible losses or profile changes that was not possible with the regularly used acidic gel-buffer system based separation method, because of the presence of the large fluorophore peak.

2. Materials and Methods

2.1. Materials

The human IgG, ribonuclease B, bovine fetuin, sodium-cyanoborohydride (1 M in THF), aminopyrenetrisulfonate (APTS) and dithiothreitol (DTT) were from Sigma-Aldrich (St. Louis, MO, USA). Hexanoic acid, Tris-base, glycerol, glacial acetic acid, acetonitrile (ACN), sodium dodecyl sulfate (SDS) and tetrahydrofuran were from VWR (Radnor, PA, USA). The PNGase F enzyme was made at University of Pannonia (Veszprém, Hungary) [10]. The Agencourt CleanSEQ carboxyl coated paramagnetic cleanup beads were from Bio-Science Kft (Budapest, Hungary). The M040 ladder of maltooligosaccharides was a kind gift from Grain Processing Co. (Muscatine, IA, USA) and the 30 μm ID (365 μm OD) fused silica capillary was from Polymicro Technologies (Phoenix, AZ, USA).

2.2. PNGase F mediated carbohydrate release

Ten microliters of IgG, ribonuclease B and fetuin (each 1 mg/mL) stock solutions were first denatured by the addition of 4 μL denaturing solution (400 mM DTT and 5% SDS) and incubated for 15 min at 70 °C. This step was followed by the addition of 2 μL PNGase F (1.35 mg/mL in 50% glycerol) and incubated for 2.0 h at 37 °C.

2.3. APTS labeling and purification

The released oligosaccharides and 10 μL of 1 mg/mL maltooligosaccharide ladder were fluorophore labeled by the addition of 20 μL reductive amination reaction mixture containing 1 μL of 20 mM APTS, 10% of 1 M sodium-cyanoborohydride (1 M in THF), 15% of HPLC grade water, 35% of glacial acetic acid and 40% of tetrahydrofuran, followed by incubation for 1 h at 60 °C with evaporative derivatization [11]. 100 μL of HPLC grade water was then added to the dry labeling reaction mixtures and the samples were analyzed by electroosmotic flow mediated CZE before, during and after purification using the Agencourt CleanSEQ paramagnetic cleanup beads as described in [9]. Briefly, the storage liquid was aspirated from 200 μL of magnetic bead suspension and 20 μL of unpurified sample was added. Three cleanup cycles were performed as follows: 170 μL of ACN was added to the sample containing bead suspension and mixed. The liquid was aspirated on a magnetic plate, and the beads containing the sample were re-suspended with 20 μL of water to continue with the next two cleanup steps and finally eluted with 80 μL of water. The waste liquids in the 3 washing fluid vials were evaporated at 37 °C overnight and then recovered in 80 μL of water. The unpurified samples were diluted 4-times before analysis to reach the same concentration as the purified samples.

2.4. Capillary electrophoresis

All capillary electrophoresis separations were run in a PA800 Plus instrument (Beckman Coulter, Brea, CA) with laser-induced fluorescent detection (excitation 488 nm, emission 520 nm) using 30 cm effective length (40 cm total), 30 μm ID (365 μm OD). bare fused silica (BFS) capillaries. The samples were analyzed in CZE mode using 253 mM Tris - 150 mM hexanoic acid (pH 8.1) background electrolyte. The applied electric potential was 30 kV at 20 °C, with the anode at the injection side and the cathode at the detection side (normal polarity). The separation capillary was rinsed for 3 min between runs by 0.5 N NaOH, followed by a 3-minute rinse with the separation background electrolyte and the samples were pressure injected by 1 psi/5 s. System control and data acquisition utilized the 32 Karat software (version 10.1, Beckman Coulter). Symbol nomenclature for representing the glycan structures in the Tables is from [12]. All separations were done in triplicates with the migration time and peak area reproducibilities of 1.09% RSD and 1.66% RSD, respectively.

3. Results and discussion

Purification after the fluorophore labeling step for capillary electrophoresis analysis of oligosaccharides with acidic gel-buffer system is routinely done by employing the carboxyl coated paramagnetic cleanup bead based process published in [9]. In that work, the washing step solutions were not analyzed because the profiles of the carbohydrates of interest before and after the purification process were close to identical. However, we have recently observed that the APTS labeled maltooligosaccharides before and after purification showed saddle quantitative profile differences. Therefore, the aim of this study was to shed light on some of the so far unrevealed details of the sample purification process after APTS based carbohydrate labeling. Using capillary zone electrophoresis mode in basic pH (CZE, pH 8.1) enabled purification free analysis of the labeling reaction mixture that was not possible earlier with the acidic gel-buffer system. Therefore, it seemed to be important to investigate the entire purification workflow including the washing fluid wastes. The approach exploited the reversed migration direction of the negatively charged labeling dye and the analyte molecules of interest, i.e., towards the inlet end, mediated under carefully designed electroosmotic flow conditions in normal polarity setup. In other words, the counter current electrophoretic migration of the free APTS dye and the APTS tagged sample components of interest were respectively greater and lower than that of the electroosmotic flow mobility. Implementing the above in normal polarity separation mode, the free APTS migrated out of the capillary at the inlet side by the application of the electric field, while the analyte molecules of interest were swept toward the detection zone by the electroosmotic flow. Each step of the purification process including the washing steps were closely scrutinized by CZE analysis under basic pH conditions including the APTS labeled maltooligosaccharide ladder and the N-glycans released from several standard glycoproteins.

3.1. Monitoring of the purification steps of the maltooligosaccharide ladder

To shed light on possible purification process related sample component losses or bias, we first analyzed the unpurified (trace a) and purified (trace b) APTS labeling reaction mixture of the maltooligosaccharides along with the three otherwise discarded washing solutions (traces c-e) of the cleanup workflow (Fig. 1). The basic Tris-hexanoic acid buffer system (pH 8.1) used in the analyses supported the generation of sufficient EOF level to drive the oppositely migrating negatively charged APTS-glycan samples toward the detector, but not the high electrophoretic mobility free dye. Due to the EOF driven analysis, the largest and consequently slowest effective mobility oligosaccharides reached the detection zone first, followed by their smaller, therefore, higher effective mobility counterparts. Under these conditions, the remaining non-reacted negatively charged APTS did not even enter the separation capillary, allowing uncompromised investigation of the purification process. The significant differences of the lower degree of polymerization glucose oligomers (peaks 10–14 corresponding

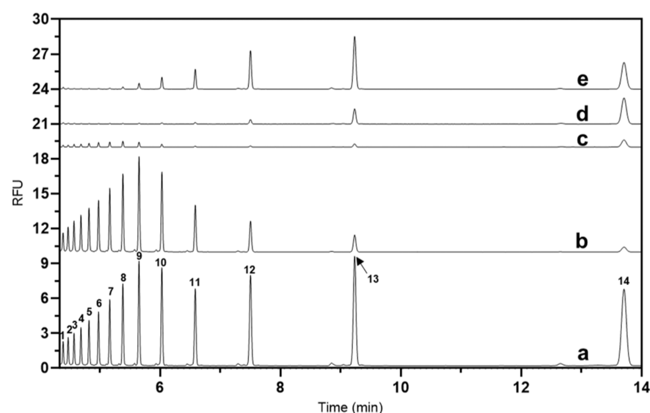

Fig. 1. Capillary zone electrophoresis analysis of APTS labeled maltooligosaccharides in pH 8.1 background electrolyte. Peaks: 1 – maltopentadecaose (GU15), 2 – maltotetradecaose (GU14), 3 - maltotridecaose (GU13), 4 – maltododecaose (GU12), 5 – maltohendecaose (GU11), 6 – maltodecaose (GU10), 7 – maltoenneaose (GU9), 8 – maltooctaose (GU8), 9 – maltoheptaose (GU7), 10 – maltohexaose (GU6), 11 – maltopentaose (GU5), 12 – maltotetraose (GU4), 13 – maltotriose (GU3) and 14: maltose (GU2). Traces: a – without purification; b – with purification; c – first washing fluid; d –second washing fluid; e – third washing fluid. Conditions: 30 cm effective/40 cm total length BFS capillary (30 µm ID); 253 mM Tris – 150 mM hexanoic acid BGE at pH 8.1; Applied electric field in normal polarity: 750 V/cm; capillary cartridge temperature: 20 °C; Pressure injection for 5 s at 1 psi.

maltohexaose to maltose) in the purified and unpurified samples are well observable between traces a and b of Fig. 1. Interestingly, the analysis of the first washing fluid revealed equal amount of sample loss with all components, probably due to the great amount of APTS dye that was removed during this step. However, later in the second and third washing steps, preferential sample components losses were observed toward the smaller size glucose oligomers (peaks 10–14 representing maltose to maltohexaose). Please note, e.g., the higher maltotriose peak in trace d compared to trace e, emphasizing cleanup process related bias during the washing steps, resulting in a total of 70% loss of this sample component after the entire purification process. On the other hand, the relative percentage changes of the higher GU fragments apparently increased up to > 90%. Table 1 depicts the quantitative peak area differences between the purified and unpurified sample traces, showing striking differences at the low GU range (peaks 12–14).

Table 1
Peak area differences between the purified and unpurified APTS labeled maltooligosaccharides (peak assignment as in Fig. 1).

Peak No.	Structure	Non purified (% Area)	Purified (% Area)	%Area change
1	GU15	1.72	2.70	57.5%
2	GU14	1.93	3.40	75.9%
3	GU13	2.24	4.18	86.7%
4	GU12	2.62	5.00	91.0%
5	GU11	3.10	5.99	92.9%
6	GU10	3.76	7.25	92.7%
7	GU9	4.71	9.01	91.3%
8	GU8	6.03	11.35	88.1%
9	GU7	8.23	14.74	79.2%
10	GU6	8.34	13.24	58.8%
11	GU5	7.32	8.70	18.9%
12	GU4	10.39	6.80	-34.6%
13	GU3	17.22	5.05	-70.7%
14	GU2	22.39	2.61	-88.4%

3.2. CZE analysis during the purification steps of APTS labeled N-glycans released from standard glycoproteins

Next the APTS labeling reaction products and washing solutions were investigated during the sample purification steps of the N-glycome analysis of some well characterized glycoproteins, including IgG, ribonuclease B and fetuin, representing complex, high mannose and highly sialylated carbohydrate types, respectively. Fig. 2 compares the unpurified (trace a) and purified (trace b) IgG N-glycan pool, along with the electropherograms of the three consecutive washing fluids (traces c-e). Table 2 shows the peak area differences between the labeled peaks in the purified (b) and unpurified (a) sample traces. By comparing traces a and b, the peaks with the longest migration times (#24–25), in this case representing the smallest and/or highly sialylated glycans, practically disappeared during the purification process. Due to their cleanup mediated disappearance, these glycans were not identified earlier in any CE databases, therefore, no structures could be assigned for them in Table 2. The ratio of some of the other sialylated carbohydrates in the IgG N-glycan pool also decreased up to 12% (peaks 21–23) as Table 2 depicts. The electropherogram of the first washing fluid was similar to the original IgG N-glycome profile, while the second and third washing fluids resulted in greater peaks in trace e than that of in trace d, suggesting non-predictable loss of some of the sample components, apparently independent of the actual washing step.

The high mannose type N-glycosylation of ribonuclease B was examined next and the results are shown in Fig. 3 with the same trace assignment as above. Here at the first sight, the unpurified and purified traces looked quite similar (traces a and b). While, the first washing fluid (trace c) contained a significant amount of all sample components with similar distribution as in the unpurified sample (trace a), the second washing solution (trace d) showed very biased distribution towards the larger mannose 8 and mannose 9 components (peaks 1 and 2). Albeit the third washing fluid apparently did contain too much of the sample components, the difference between the first and the second washing fluid content already suggested some profile bias caused by the purification process as depicted in the peak area values in Table 3. For example, the loss in the Man 9 structure was as high as 16.4% and different level of losses were observable for the other components. As a conclusion, the purified profile did not represent the original quantitative distribution of the high mannose sample components.

The highly sialylated fetuin N-glycans also revealed subtle profile differences apparently caused by the purification process, as shown in Fig. 4 by comparing traces a and b. Most strikingly, the peak area ratios swapped between the two major components of A3G3S(3)2S(6)1 and A3G3S(3)1 S(6)2 during the purification process. The

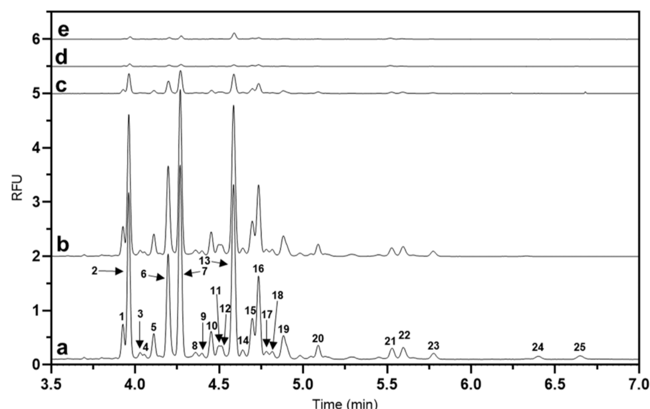

Fig. 2. Capillary zone electrophoresis analysis of PNGase F released and APTS labeled IgG N-glycans. Peaks: see Table 2. Traces: a – without purification; b – with purification; c – first washing fluid; d –second washing fluid. e – third washing fluid. Conditions: same as in Fig. 1.

Table 2
Peak area differences between the purified and unpurified APTS labeled IgG N-glycans. (peak assignment is based on [13]).

Peak No.	Structure	Non purified (% Area)	Purified (% Area)	%Area change	Peak No.	Structure	Non purified (% Area)	Purified (% Area)	%Area change
1	FA2BG2	2.94	3.18	8.23%	14	A2B	1.23	1.30	5.21%
2	FA2G2	13.44	13.37	-0.53%	15	FA2BG2S1	4.92	4.88	-0.83%
3	FA2B(3) G1	0.81	0.84	3.35%	16	FA2G2S1	8.49	8.54	0.64%
4	A2G2	0.58	0.82	42.45%	17	A2BG2S1	0.99	1.03	4.42%
5	FA2(3)G1	2.48	2.50	0.63%	18	A2G2S1	0.98	0.92	-5.77%
6	FA2(6)G1	10.09	10.28	1.87%	19	FA2(3) G1S1	3.97	4.04	1.76%
7	A2B(3)G1	17.39	17.72	1.91%	20	FA2(6) G1S1	1.66	1.66	0.46%
8	A2B(6)G1	0.99	1.10	10.99%	21	FA2BG2S2	1.57	1.37	-12.72%
9	A2B(6)G1	0.76	0.76	0.00%	22	FA2G2S2	1.73	1.62	-6.41%
10	FA2B	2.76	2.83	2.46%	23	A2G2S2	0.93	0.87	-5.93%
11	A2(6)G1	1.29	1.41	9.57%	24	unknown	0.57	0.00	-100.00%
12	A2(6)G1	1.30	1.25	-4.04%	25	unknown	0.69	0.00	-100.00%
13	FA2	17.45	17.77	1.83%					

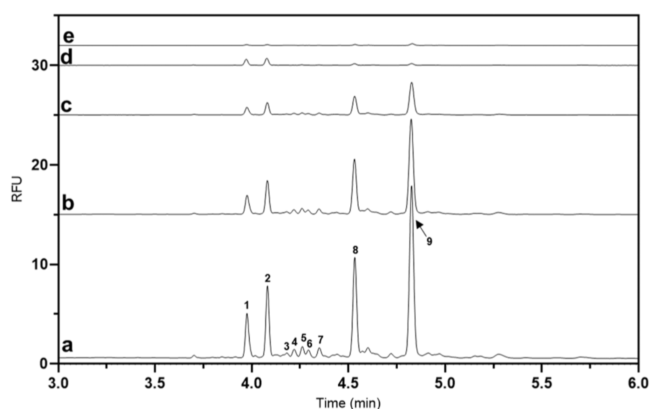

Fig. 3. Capillary zone electrophoresis analysis of PNGase F released and APTS labeled Ribonuclease B N-glycans. Peaks: see Table 3. Traces: a – without purification; b – with purification; c – first washing fluid; d –second washing fluid. e – third washing fluid. Conditions: same as in Fig. 1.

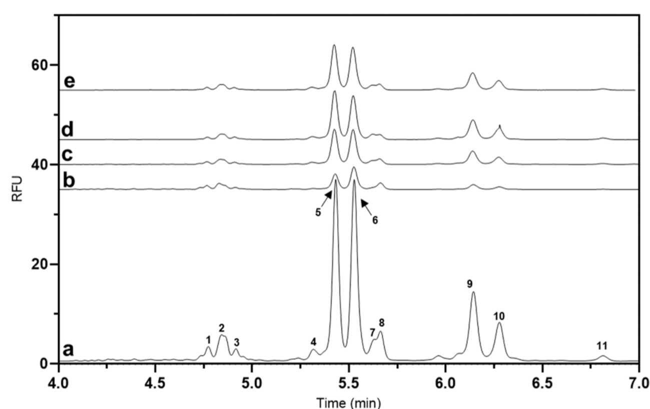

Fig. 4. Capillary zone electrophoresis analysis of PNGase F released and APTS labeled fetuin N-glycans. Peaks: see Table 4. Traces: a – without purification; b – with purification; c – first washing fluid; d –second washing fluid. e – third washing fluid. Conditions: same as in Fig. 1.

Table 3
Peak area differences between the purified and unpurified APTS labeled Ribonuclease B N-glycans (peak assignment was based on [14]).

Peak No.	Structure	Non purified (% Area)	Purified (% Area)	%Area change
1	Man 9	9.86	8.24	-16.4%
2	Man 8c	14.25	12.92	-9.3%
3	Man 8b	1.10	1.02	-6.5%
4	Man 8a	1.99	1.98	-0.6%
5	Man 7c	2.35	2.29	-2.6%
6	Man 7b	1.78	1.77	-0.8%
7	Man 7a	2.39	2.28	-4.5%
8	Man 6	22.92	23.91	4.3%
9	Man 5	43.37	45.58	5.1%

electropherograms of the washing fluids (traces c-e) all showed significant amounts of all sample components at slightly changing distribution, probably causing the profile bias between the purified and non-purified sample as depicted in Table 4.

4. Conclusions

In this paper we investigated the purification process steps after APTS labeling of carbohydrates to shed light on qualitative and/or quantitative separation profile bias due to sample components possibly washed away during the cleanup process. Capillary zone electrophoresis

Table 4
Peak area differences between the purified and unpurified APTS labeled fetuin N-glycans (peak assignment was based on [15]).

Peak No.	Structure	Non purified (% Area)	Purified (% Area)	%Area change
1	A3G3S(3)2 S(6) 1*	2.01	4.83	139.9%
2	A3G3S(3)1S(6) 2*	4.28	7.33	71.5%
3	A3G3S(6)3*	1.97	2.59	31.7%
4	A3G3S(3)2 S(6) 1	2.95	1.42	-51.8%
5	A3G3S(3)2S(6) 1	28.63	22.96	-19.8%
6	A3G3S(3)1 S(6) 2	29.87	33.37	11.7%
7	A3G3S(3)1 S(6) 2**	3.76	3.31	-12.0%
8	A3G3S(6)3	3.96	8.65	118.6%
9	A3G3S(3)2 S(6) 2	13.90	8.97	-35.5%
10	A3G3S(3)1 S(6) 3	7.90	4.76	-39.7%
11	A3G3S(3)2 S(6) 3*	0.78	0.23	-70.1%

* GlcNAc (1,4 or 1,2) linkage
** positional isomers [15]

mode using the basic pH Tris-hexanoic acid background electrolyte enabled us to analyze the samples without any interference by the remaining fluorophore labeling reagent in the reaction mixture. The online sample cleanup took advantage of the thoughtfully designed electroosmotic flow mobility and the oppositely migrating higher and lower respective effective mobilities of the remaining free APTS and sample components of interest. Using this separation mode allowed to reveal the amount and type of sample components apparently lost at different levels during the sample purification process. The quantitative profile bias towards the smaller oligosaccharides was already well visible by analyzing the maltooligosaccharide ladder. Analysis of the APTS labeled N-glycan pools of several well characterized glycoproteins (IgG, ribonuclease B and fetuin) also revealed unexpected qualitative and quantitative sample component losses during the washing steps. Also important to note that the average standard deviation of the percentage area of randomly selected peaks in three parallel cleanup steps was close to 4% RSD, suggesting low reproducibility of the purification process itself. Finally, although, EOF driven separations were not previously recommended because of the not evenly spaced peaks at higher GU level, our data contradistinctionally demonstrated the advantage of EOF driven capillary zone electrophoresis separation at basic pH with oppositely migrating analyte molecules in an easy to use purification-free workflow. This method would also be advantageous to recognize possible sample losses during other commonly used carbohydrate purification processes, where the opposite migration of the labeling dye and the sample component is not viable for online sample cleanup.

CRediT authorship contribution statement

Robert Farsang: Formal analysis, Conceptualization, Methodology. **Gabor Jarvas:** Validation. **Andras Guttman:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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