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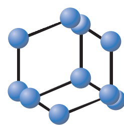

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Evaluation of Possible Processing Time Effects on the Global *N*-Glycosylation Profile of Human Blood Samples

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Abstract: The utilization of *N*-glycan profiling recently gained high importance in fundamental biomedical and applied clinical research. However, for the time being, no glycan biomarker has been approved for clinical diagnosis by the regulatory agencies due to the lack of verifications on large patient cohorts and suitable analytical technologies. In this paper, the effect of human blood sample handling was studied prior to *N*-glycosylation profiling by capillary electrophoresis, coupled with high sensitivity fluorescence detection. Special attention was paid to the preservation of sialylated structures because of their important clinical - biological relevance. Our results suggested that it is adequate to refrigerate and store the collected total blood samples prior to analysis to obtain unbiased results. Furthermore, we report on the good practice of serum sample handling in order to prevent decomposition of the sialylated structures. Our findings may promote procedure standardization and easier clinical translation of diagnostic *N*-glycosylation profiling in molecular medicinal applications.

Keywords: Blood sampling, storage temperature, storage time, preanalytical processing, *N*-glycome, capillary electrophoresis.

1. INTRODUCTION

Solid evidence has been reported in the literature that the *N*-glycosylation of blood-born glycoproteins may be altered by various diseases [1, 2]. Thus, utilization of *N*-glycan profiling from liquid biopsy samples, such as blood, for diagnostic purposes gained high importance both in fundamental biomedical and applied clinical research. Asparagine linked glycosylation is reportedly affected by various diseases, resulting in aberrant glycosylation on most glycoproteins in human blood [1]. A comprehensive analysis of these changes can be utilized for diagnostic purposes.

Carbohydrates are among the most structurally diverse species in nature and are usually analyzed by high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS) or the hyphenation of those, such as LC-MS or CE-MS [3]. One of the most studied subsets of aberrant glycosylation vs disease state is cancer [4] in spite of the fact that for the time being, none of the potential glycan biomarkers have been approved for clinical diagnosis by the regulatory agencies [5]. Successful clinical translation is restrained by the lack of verifications on large patient cohorts and suitable fast, efficient and high throughput analytical technologies. In addition, albeit the methods used for analytical glycomics are well developed, their integration into complete analytical workflows is still in progress.

Multiple factors should be considered during method development and validation to achieve good analytical efficiency, resolution, accuracy, high throughput, and robustness. Individual variability of the *N*-glycome regarding sample collection time and condition (*e.g.*, storage temperature) as well as factors during sample preparation are of high importance. Details in analytical processes and standardization of data evaluation are all very important too. In an earlier study, a great variability of individual glycan profiles was observed in a population of 1008 volunteers [6]. This demonstrated the need for appropriate sample preparation protocols and the standardization of adequate glycoanalytical tools for carbohydrate profile and composition analysis. For example, the *N*-glycan composition of blood samples is subject to change due to the presence of enzymes, even the ones that do not require ATP for their action [7-11]. Enzyme activity can be affected by a variety of factors, such as temperature, pH, and concentration of substrates, just

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to mention a few [12]. While the importance of sample handling and preservation have been reported for blood-based proteomics [13-15] and metabolomics [16, 17] studies, similar evaluation is greatly needed for glycomics. Dedova et al. published a pioneering paper on the effect of pre-analytical conditions on single glycan structure determination as well as on glycan-based ovarian cancer biomarker GLYCOV scoring [18]. The authors investigated the effect of storage temperature and processing time on the glycan profiles of 10 healthy volunteers after desialylation. However, it is important to note that sialylated structures often hold important biological information [2], which are lost if the samples are desialylated. Ventham and coworkers studied the effect of different sample tubes and processing methods on whole serum *N*-glycan profiles and found that the investigated factors caused minimal inter-individual variations [19]. Cadamuro et al. came to the same conclusion, namely that the centrifugation conditions did not alter the analyte composition tested in their study [20]. Interestingly, even otherwise easily overlooked parameters such as the sample volume should be considered when assessing the hydration status of human blood [21].

One of the most important aspects of any clinical laboratory analysis is the choice of biospecimen processing, *i.e*., preparation of samples (e.g., blood) after collection. Whole blood, serum, plasma, buffy coat, and peripheral blood mononuclear cells are the common options to be considered as well as the subsequent pre-analytical processing methods and conditions including centrifugation, storage temperature, process duration, *etc*. [22]. For global *N*-glycosylation profile analysis, human serum is suitable because it can be analyzed without the interference of insoluble fibrin aggregates or cross-contamination from anticoagulants [23].

In this work, the effect of human blood sample handling was studied prior to *N*-glycosylation profiling by capillary electrophoresis, coupled with high sensitivity fluorescence detection. Special attention was paid to the preservation of sialylated structures because of their important clinical – biological relevance.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Water (HPLC grade), acetonitrile, sodium cyanoborohydride (1 M in THF), glycerol, ammonium acetate and DTT (dithiothreitol) were obtained from Sigma Aldrich (St. Louis, MO, USA). SDS (sodium dodecyl sulfate) and Nonidet P-40 were from VWR (Radnor, PA, USA). The Fast Glycan Labeling and Analysis Kit was from SCIEX (Brea, CA, USA), including the 8-aminopyrene-1,3,6-trisulfonic acid (APTS) labeling reagent, the bracketing standard (containing maltose, DP2 and maltopentadecaose, DP15) and the magnetic beads. The endoglycosidase PNGase F was from Asparia Glycomics (San Sebastian, Spain). EDTA was from Alfa Aesar (Ward Hill, MA, USA).

2.2. Specimen Collection and Sample Preparation

Capillary blood samples were collected from a healthy female (ethical approval UD 5570-1/2018/EKU, and informed patient consent). Each sample was taken simultaneously in the morning using blood lancets (Vitrex Medical, Herlev, Denmark). 1.2 µl of 3.19 mM EDTA anticoagulant was added to 2 µl collected total blood sample and diluted by adding 96.8 µl HPLC water in 200 µL vial. The collected total blood samples were processed in five main steps: denaturation, digestion, glycan release, fluorophore labeling and magnetic bead mediated cleanup. Six samples were kept at room temperature for 0, 60, 120, 180, 240 and 300 minutes and then refrigerated at 4 °C for 24 hours. Another six samples were kept at room temperature for 0, 60, 120, 180, 240 and 300 minutes and then immediately diluted with HPLC grade water to 97 µL for further processing.

Prior to *N*-glycan release, the samples were denatured at 80 °C for 10 minutes in a heating block. Release of the *N*-glycans from the denatured proteins was performed by adding 1.0 µL of PNGase F (200 mU), and incubated at 50 °C for 20 minutes. Then, evaporative fluorescent labeling was performed using 4.0 µL of 40 mM APTS in 20% acetic acid, 2.0 µL of $NaBH_3CN$ (1 M in THF) and 4 µL 20% acetic acid as described earlier [24]. The reaction mixture was then incubated in a heating block with open cap at 37 °C overnight under fume hood. After the labeling step, the samples were purified by magnetic beads following the Fast Glycan Sample Preparation and Analysis protocol (SCIEX) and analyzed by CE-LIF.

Serum samples were collected from healthy volunteers. Vacutainer blood sampling tubes (filled with clot activator and separator gel) were from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). The serum samples were collected in tubes containing the clot activator and the separator gel. Each sample was kept at room temperature for 30, 90, and 270 minutes in order to evaluate the possible effect of delayed centrifugation. The control samples were centrifuged immediately after collection, typically within 30 minutes. Centrifugation was performed in 2690 x g at 4°C for 20 minutes. After the centrifugation, samples were aliquoted into triplicates and stored at - 20°C until further processing.

Serum samples were processed as follows: 2 µL of serum was diluted with HPLC grade water to 10 µL, followed by temperature gradient denaturation by increasing the temperature with the rate of 5.0°C/min from 30°C to 80°C with an additional 5.0 min incubation step at 80°C with 5 µL denaturation solution containing 0.375 % NP-40, 12.75% glycerol, 0.625% SDS and 12.5 mM DTT as reported previously [25]. Release of the *N*-glycans from the denatured proteins was performed by adding 1.0 µL of PNGase F (200 mU) and 19 µL water, followed by incubation at 50°C for 60 minutes. The samples were then cooled in ice for 1

minute, diluted with 70 µL of ice-cold acetonitrile in order to precipitate the protein content by centrifugation at 11.290 x g for 10 minutes. The released glycan containing supernatant was dried in a vacuum centrifuge and labeled using the evaporative labeling approach and purified as described above.

2.3. Capillary electrophoresis

Capillary electrophoresis analyses with laser induced fluorescent detection (CE-LIF) (excitation:488 nm, emission: 520 nm) were performed using a PA800 Plus Pharmaceutical Analysis System (SCIEX). Separation conditions for whole blood samples: 30 cm total (20 cm effective) length, 50 µm i.d. bare fused silica capillary with HR-NCHO gel buffer (Sciex); Separation voltage: 30 kV (0.5 min ramp) in reversed polarity mode (cathode at the injection side, anode at the detection side); temperature: 25°C; injection: 5 psi/5 sec water pre-injection followed by 2 kV/2 sec sample. Separation conditions for serum samples: 60 cm total (50 cm effective) length, 50 µm i.d. bare fused silica capillary with NCHO gel buffer (Sciex); Separation voltage: 30 kV (0.5 min ramp) in reversed polarity mode; temperature: 25°C; injection: 1 psi for 5 sec. In both cases, data acquisition and analysis were performed by the 32Karat (version 10.1) software package (SCIEX). Relative peak area percentage values of the separated components were calculated by the PeakFit v4.12 Software (SeaSolve Software Inc., San Jose, CA). GU value calculation and structural *N*-glycan assignment were accomplished by using the GUcal software and its built-in database (www.gucal.hu) [26].

3. RESULTS AND DISCUSSION

In this study, the effect of processing conditions on the global *N*-glycosylation profile of human blood samples was investigated by CE-LIF with special attention on the fate of sialylated structures. Recently reported new sample preparation protocols were applied to accommodate the high complexity of the human blood *N*-glycome, including temperature adjusted denaturation and evaporative labeling protocol [24, 25]. First, the global *N*-glycosylation profile of whole human blood was analyzed in as native form as rationally possible in order to establish a reference state for the consequent comparisons [26]. The most commonly used anticoagulant in clinical practice is heparin, a naturally occurring glycosaminoglycan, thus may cross-contaminate sensitive CE based glycan profiling [27, 28]. To avoid the appearance of heparin originated glycans in the blood *N*-glycan profiling, ethylenediamine tetraacetic acid (EDTA) was used instead of heparin [29]. EDTA was added to the collected whole blood samples and immediately processed in order to minimize any possible changes in the *N*-glycan composition by chemical or enzymatic degradation. A representative electropherogram of the healthy human total blood sample *N*-glycome is shown in Fig. **1**. Structural elucidation of all separated *N*-glycans was accomplished as reported in [30]. Briefly, structure identification utilized direct mining of GU database entries (GUcal application linked with the GlycoStore data collection), and some earlier published literature data on similar samples [30-32].

After establishing the reference profile, the sample handling conditions have been studied. In clinical environments (among others, local medical core-facility laboratories and global serving testing laboratories) where numerous blood samples are analyzed daily, immediate sample processing is not always feasible, thus storage could not be avoided. Therefore, the effect of storage time and temperature on the *N*-glycosylation profile of the collected whole human blood was investigated. One set of sample was stored at 4°C for 24-hour before sample processing and analysis, while another set was processed immediately after blood collection. The resulted electropherograms are compared using a mirror diagram shown in Fig. **2**. Trace A depicts the CE-LIF *N*-glycan profile of the blood sample kept at room temperature for 2 hours, then stored at 4°C for 24-hour before sample processing. Trace B shows the results when the sample was kept at room temperature for 2 hours and immediately processed without any further storage. Peaks #1-4 correspond to variously sialylated structures showing only minor changes during the 24 hour 4°C storage. It is important to note that sialylated structures tend to be sensitive to small environmental alterations [2, 33]. Peaks #5-13 revealed no apparent change. Peaks marked with asterisks were not identified but showed significant changes in the *N*-glycan profile due to temperature depending degradation. This degradation of the sugar oligomers was earlier assumed to be the result of non-ATP mediated enzymatic reactions, which might still work under extracorporeal conditions [10]. The presumable mannose oligomers are marked with an asterisk in Fig. **2**. As a conclusion of these experiments, it seems to be adequate to store the collected total blood samples at 4°C for 24 hours in order to ensure unbiased results of the applied sample preparation protocol and CE-LIF separation. In order to alleviate the reproducibility issues of the CE separations, peaks were normalized to peak 13.

Since immediate cooling after blood collection is often not possible due to practical reasons, the samples are kept at room temperature for a certain period of time. Thus, we investigated the effect of room temperature storage as the function of elapsed time. Collected samples were kept for 0, 1, 2, 3, 4, and 5 hours at 22°C, then stored for 24 hours at 4°C before processing. The resulted separation traces are compared in Fig. **3**. A thorough inspection of the traces suggested that up to five hours of room temperature storage no significant alteration occurred in the total *N*-glycome profile of the whole blood sample. Most importantly, even the sialylated structures were not affected by these storage conditions, as evidenced by the unchanged profiles of peaks 1-5.

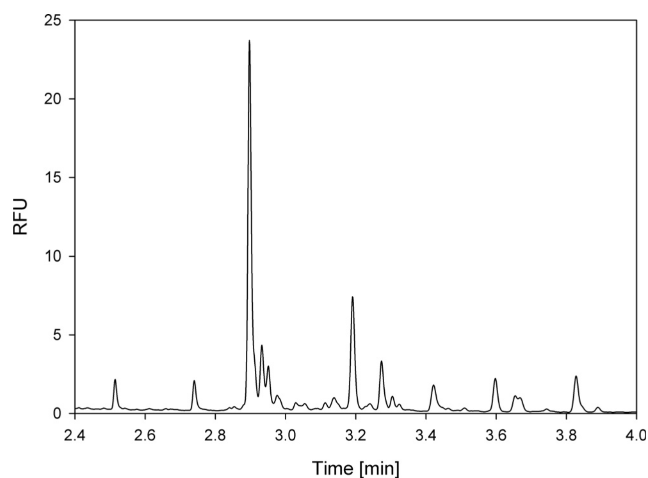

Fig. (1). Capillary electrophoresis separation of the APTS-labeled human whole blood *N*-glycome. Anticoagulation of the collected sample was achieved by the addition of EDTA and the specimen was processed immediately after collection. Separation conditions: 30 cm total (20 cm effective) length, 50 µm i.d. bare fused silica capillary with HR-NCHO gel buffer. Voltage: 30 kV (0.5 min ramp); Temperature: 25°C; Injection: 5 psi/5 sec water pre-injection followed by 2 kV/2 sec sample. Structures corresponding to peaks: 1: A2G2[6]S2 2: A2BG2S2 / M3 3: FA2BG2S2 4: A2G2[6]S1 5: FA2G2S1 6: FA2 7: M6 8: FA2[6]G1 9: M7 10: FA2[3]G1 11: FA2B[6]G1 12: M8 13: FA2G2. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

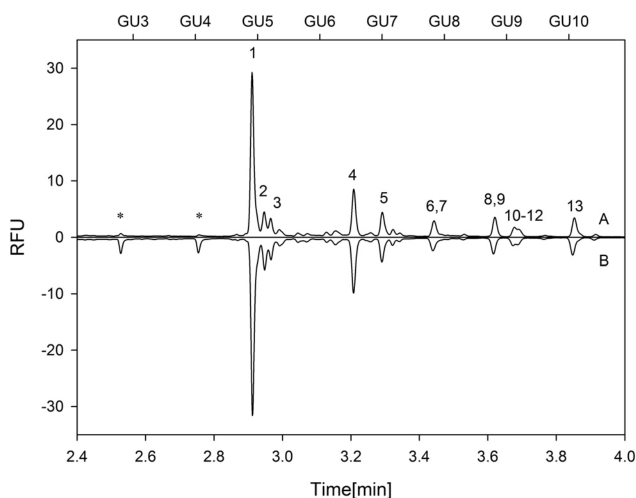

Fig. (2). Capillary electrophoresis mirror diagram of the APTS-labeled human blood N-glycome from a blood sample kept at room temperature for 2 hours then stored at 4°C for 24-hour before sample processing (A) and from a sample that was kept at room temperature for 2 hours then processed immediately (B). Separation conditions and structures were the same as in Fig. **1**. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

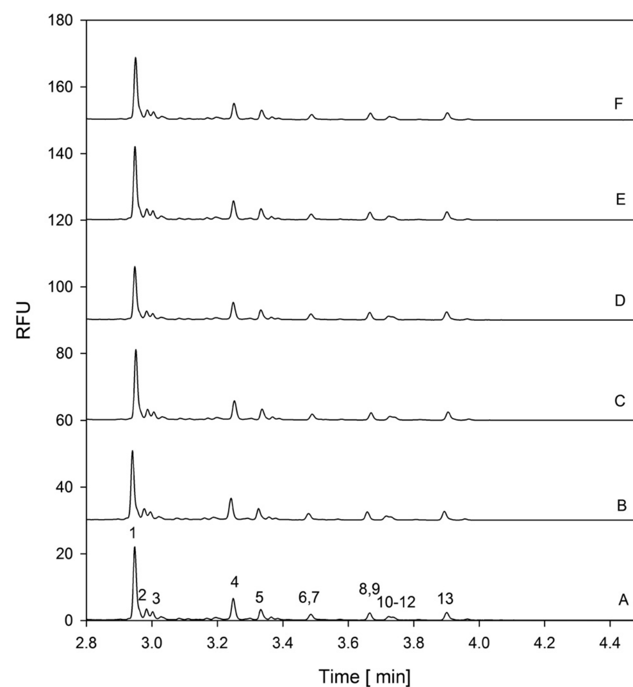

Fig. (3). Comparative capillary electrophoresis analysis of the APTS-labeled human blood N-glycome as a function of room temperature storage time. The specimens were kept for (A) 0; (B) 1; (C) 2; (D) 3; (E) 4 and (F) 5 hours at 22 °C after blood taking, then stored for 24 hours at 4°C before processing. Separation conditions and structures corresponding to the separated peaks were the same as in Fig. **1**. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Next, the determination of the appropriate reference state and its storage conditions was assessed using the standard procedure for total serum *N*-glycome profiling. In this stage of the study, conventionally used serum sample tubes were employed to explore the robustness of the protocol with special attention on conserving the sialylated structures. Serum samples were obtained by collecting whole blood from healthy volunteers in sampling tubes containing clot activator and separator gel (Table **1**). Each sample was kept at room temperature for 1.5 and 4.5 hours in order to evaluate the possible effect of delayed centrifugation, which is the first step of the standard serum processing workflow. The control sample was centrifuged after collection, typically within 30 minutes, considering all necessary steps for serum processing after taking the blood sample. After centrifugation, the samples were stored at - 20°C until further processing. The resulted *N*-glycan profiles of the serum samples are compared in Fig. **4**. Traces A, B and C demonstrate the effect of different elapsed times prior to centrifugation.

Please note that traces were normalized to peak 13 in this case as well. Sialylated structures (peaks 1-5) were significantly decreased by 4.5 hours of storage time at room temperature. In addition, the ratio of peak

4 relative to peak 3 increased. The degradation of the sialic acid containing structures can be explained by the fact that these residues are sensitive to any minor changes such as storage duration and temperature. As one can observe in Fig. **4**, non-sialylated glycan structures (peaks 6-13) remained stable during the investigated time period.

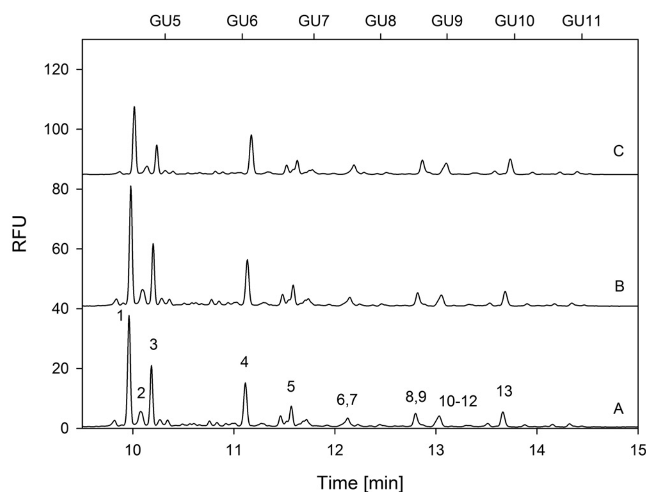

Fig. (4). Comparative capillary electrophoresis analysis of the APTS-labeled human serum N-glycome. The specimens were kept for (A) 0.5 (control); (B) 1.5; (C) and 4.5 hour at room temperature after blood taking then centrifuged for 20 min at 4°C before processing. Separation conditions: 60 cm total (50 cm effective) length, 50 µm i.d. bare fused silica capillary with NCHO gel buffer. Voltage: 30 kV (0.5 min ramp); Temperature: 25°C; Injection: 1 psi for 5 sec. Structures corresponding to peaks are the same as in Fig. **1**. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

CONCLUSION

In this work, the effect of human blood sample handling was studied prior to *N*-glycosylation profiling by capillary electrophoresis coupled with high sensitivity fluorescence detection. Two different biospecimens (total blood and serum samples) were studied investigating the influence of storage duration and temperature conditions. The total blood was processed utilizing recently reported new sample preparation protocols, including temperature adjusted denaturation and evaporative labeling to accomplish *N*-glycome profile determination in its native form. Profile of the total blood was used as a reference for the subsequent comparative studies. In contrast, serum samples were analyzed using conventional workflows to explore the robustness of the currently applied techniques with special attention to conserving the sialylated structures.

Human whole blood *N*-glycome profile from capillary electrophoresis separation was reported together with the structural elucidation of the most significant components using direct mining of GU database entries.

Table 1. Identified *N*-glycan structures (nomenclature followed the guidelines of the Consortium of Functional Glycomics [34]). Entry numbers correspond to the peak numbers in the Figures. Symbols: ◆ Sialic acid (*N*-acetylneuraminic acid); ● Galactose; ■ *N*-acetylglucosamine; ● Mannose; ▼Fucose.

Peak No.	Glycan name	Glycan structure
1	A2G2S2	
2	A2BG2S2	
	M3	
3	FA2BG2S2	
4	A2G2S1	
5	FA2G2[3]S1	
6	FA2	
7	M6	
8	FA2[6]G1	
9	M7	
10	FA2[3]G1	
11	FA2B[6]G1	
12	M8	
13	FA2G2	

Our results of the storage condition assessment suggested that it was adequate to refrigerate and store the collected total blood samples up to 24 hours prior to analysis and still obtain apparently unchanged profiles. However, immediate cooling after blood collection is often not possible. Thus we investigated the effect of room temperature storage on the total *N*-glycome profile of the whole blood samples. No significant alteration was found in the total *N*-glycome; even the sialylated structures were conserved.

Finally, we investigated the effect of the processing time on the global *N*-glycosylation profile of human serum samples collected by the standard clinical protocol (i.e., utilization of blood sampling tubes filled with clot activator and separator gel). It was concluded that the sialylated structures were conserved up to 1.5 hours. However, it has been demonstrated that longer than 1.5 hours of storage at room temperature, the sialylated structures started to decompose. After 4.5 hours of storage time, the overall amount of sialylated structures decreased by ~50 %. As a conclusion, it is strongly recommended to avoid keeping the drawn blood samples for more than 1.5 hours at room temperature prior to centrifugation. It is especially important when samples are intended to be used for *N*-glycosylation profiling including sialylated structures.

The reported findings demonstrated that the blood *N*-glycome is a robust target for molecular medicinal research provided not to be stored at room temperature for longer than 1.5 hours. Based on the results reported in this paper, we plan to carry out future studies involving a larger cohort of samples from different patient groups for statistical purposes.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval obtained from EKU with approval No. UD 5570-1/2018/EKU.

HUMAN AND ANIMAL RIGHTS

Humans were used for this study.

CONSENT FOR PUBLICATION

Written inform consent was obtained from on all patient.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors disclose no conflicts.

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