Procainamide fluorescent labeling for identification of low abundant non-invasive glycan biomarkers

Zsuzsanna Kovács[1], Yongjing Xie[2], Barbara Adamczyk[1], Fiona Reidy[3], Fionnuala M. Mcauliffe[4], Pauline M. Rudd[1,5], Michael Butler[2], Mary Wingfield[3,4], Louise Glover[3], Radka Saldova[1,5]

[1] NIBRT GlycoScience Group, National Institute for Bioprocessing Research and Training, Belfield, Blackrock, Co. Dublin, Ireland; [2] NIBRT Cell Technology Group, National Institute for Bioprocessing Research and Training, Belfield, Blackrock, Co. Dublin, Ireland; [3] Merrion Fertility Clinic and National Maternity Hospital, Dublin, Ireland; [4] UCD Perinatal Research Centre, School of Medicine, University College Dublin, National Maternity Hospital, Dublin, Ireland
[5] UCD School of Medicine, College of Health and Agricultural Science (CHAS), University College Dublin (UCD), Dublin, Ireland

Background

There are limited publications on endometriosis and glycosylation, and most of the studies are conducted with tissue or peritoneal fluid samples, collected by invasive means. An Iraqi study draws attention to the importance of serum sialylation, which is dramatically changed in endometriosis patients after zoladex therapy (1), indicating that changes in serum sialylation may be a new biomarker of the disease.

While glycosylation of urine in endometriosis has not been studied so far, in a study of endometrial cancer, the urinary level of two glycoproteins was significantly increased in the patients compared to the control group. A prospective study was designed in collaboration with Merrion Fertility Clinic (MFC), in which serum and urine samples were collected for glycome analysis in women with and without endometriosis, as diagnosed at laparoscopy. The study was approved by the Research and Ethics Committee of the National Maternity Hospital, Dublin (EC19.2018). *N*-glycans from all serum and urine glycoproteins and serum IgG were analyzed using hydrophilic interaction liquid chromatography-ultra performance liquid chromatography (HILIC-UPLC).

Objective

The aim of the collaborative work was to investigate the *N*-glycosylation of whole serum glycoproteins and IgG.

Furthermore, we were interested in urinary *N*-glycosylation as a potential source of new non-invasive biomarkers. The first step was to develop an effective, reproducible method for urine processing. The study of urinary *N*-glycosylation has been neglected in the literature, despite the fact that urine can be collected in a simple, harmless, and non-invasive manner.

Materials & Methods

N-glycans on total serum glycoproteins, serum IgG and total urine glycoproteins from all samples (24-controls without endometriosis, 27 patients with mild and 27 with severe stages of endometriosis) were separated using HILIC-UPLC.

N-glycans from all serum glycoproteins were released from 5 µL of serum using a high-throughput method (2). The released *N*-glycans were fluorescently labelled with 2-Aminobenzamide (2AB), then the excess 2AB was removed (3). Ultra-performance liquid chromatography (UPLC) was performed using a HILIC column on an Acquity UPLC. Five mL of urine was concentrated with centrifugation then precipitated with trichloroacetic acid (TCA). The whole protein sample was diluted with 5 µL of ultra-pure water (ddH$_2$O) then the above-mentioned protocols were performed.

Fifty µL of serum IgG was used for *N*-glycan release using the method described by Kovacs et al. (4). Ten mL of urine was concentrated then the proteins washed with ddH$_2$O and the remaining supernatant (500 µL) was used for IgG extraction and *N*-glycan release (4). For increased sensitivity, a fluorescent tag 4-amino-N-[2-(diethylamino)ethyl] benzamide (ProA) was used for labelling of the urinary IgG *N*-glycans at 65˚C, 1 hour, and the excess ProA was removed by using the above-mentioned clean-up protocol (3).

Urine *N*-glycome was analysed using HILIC-UPLC, exoglycosidase digestions and mass spectrometry.

Statistical analyses were performed in IBM® SPSS® Statistics software, in which control was compared with mild and severe disease groups. The glycan data were logit transformed and multivariate analysis of variance (MANOVA) with Tukey test was performed.

Results

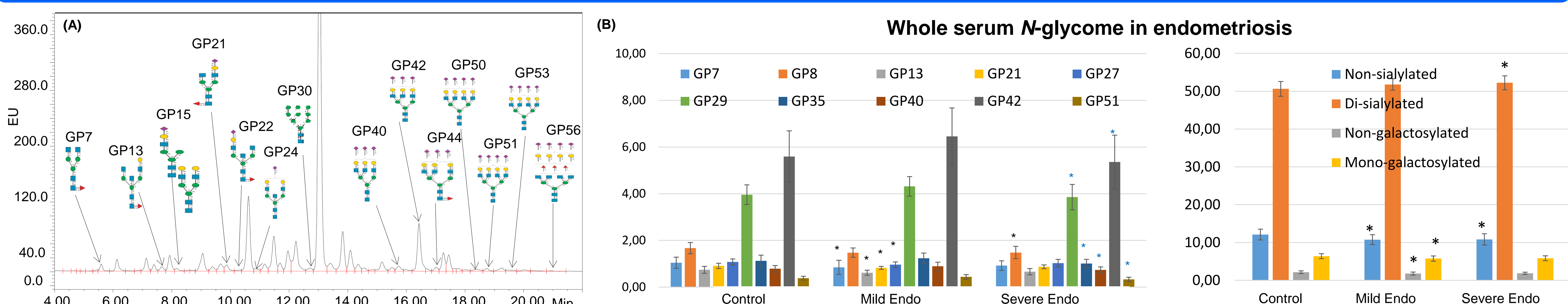
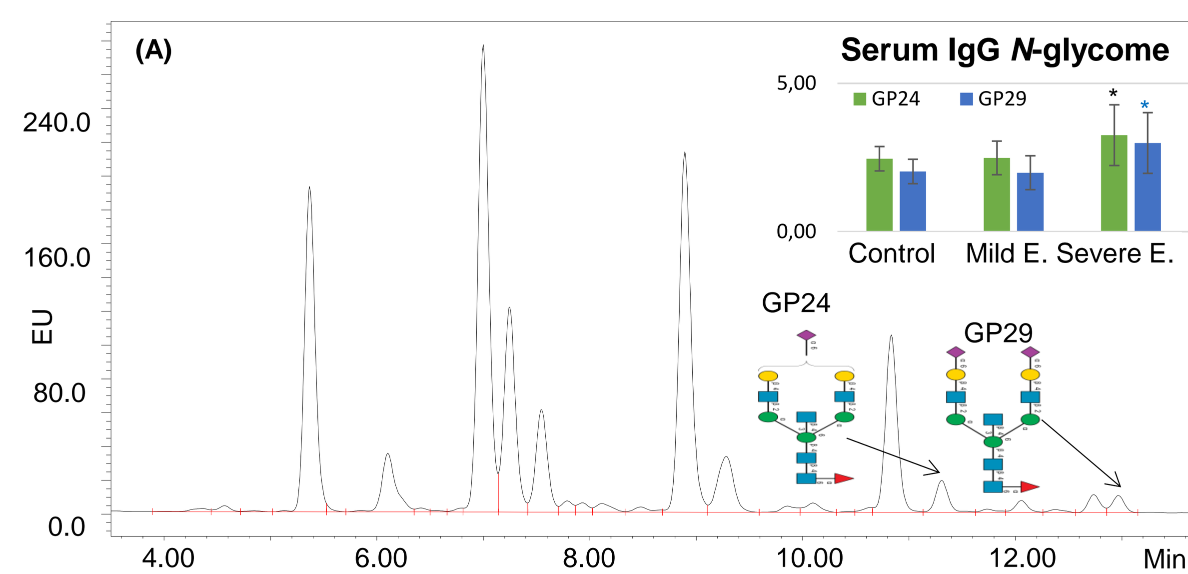


Figure 1. (A) HILIC-UPLC chromatogram of the whole *N*-glycome from a control (without endometriosis) sample and glycan peaks (GP) that are significantly changed during endometriosis. **(B)** Bar chard of GPs and features significantly altered in endometriosis. Feature analysis and the glycan composition were done according to Saldova et al. (5). The significant alteration were marked with an asterisk. *= p<0.05; **= p<0.01, black * in case of significant difference with control group, blue * in case of significant difference in severe vs mild endometriosis.

Serum and urine IgG *N*-glycome in endometriosis



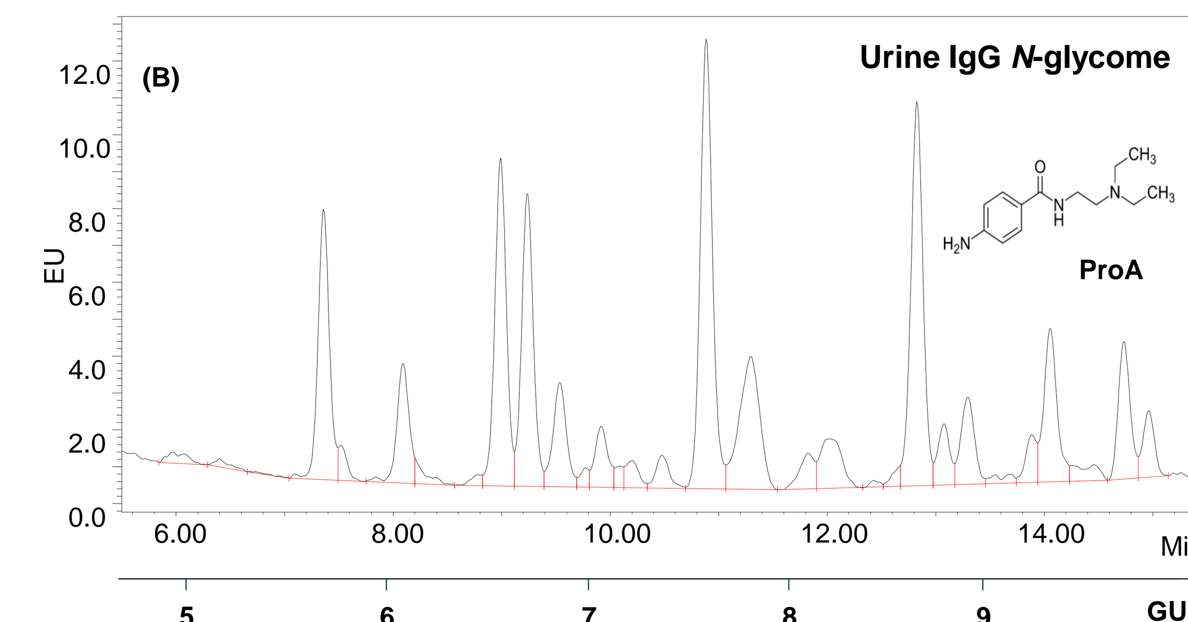
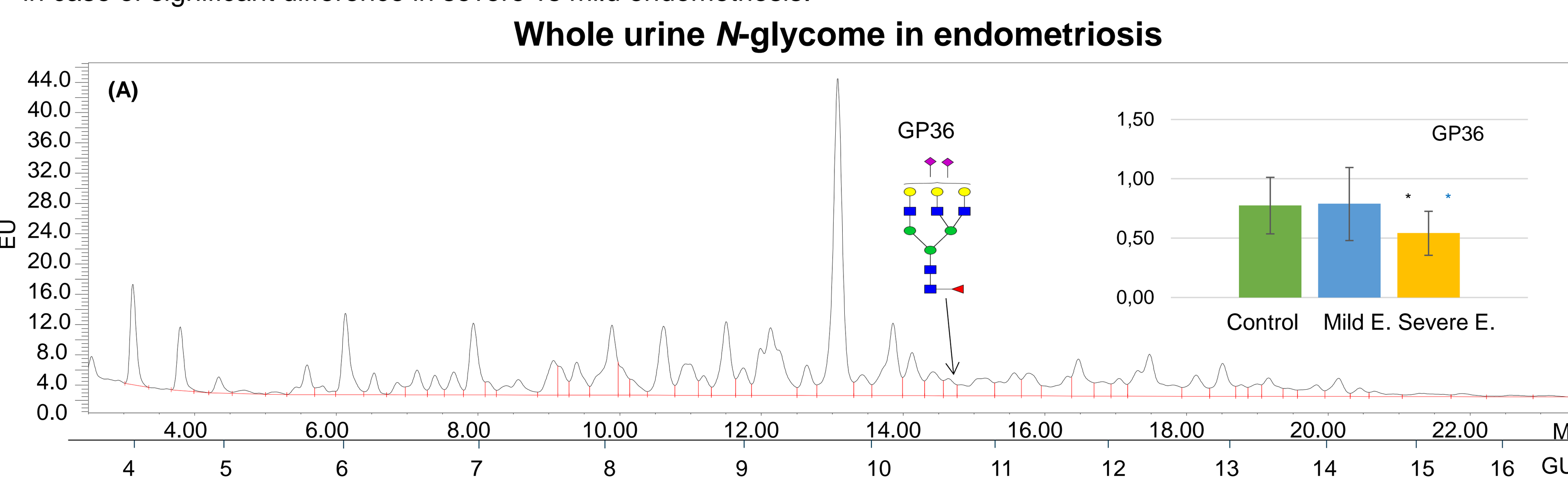


Figure 2. Representative HILIC-UPLC chromatograms of IgG *N*-glycan profile of control (without endometriosis) sample from (**A**) serum and (**B**) urine. Released IgG *N*-glycans were labelled with ProA, its schematic picture is presented. GPs which were significantly altered in endometriosis are highlighted. IgG glycan composition assignments were done according to Pucic et al. (6). The significant alterations between the control group vs mild and severe endometriosis were plotted and marked with an asterisk. *= p<0.05; **= p<0.01, black * in case of significant difference with control group, blue * in case of significant difference in severe vs mild endometriosis.

Structure abbreviations: All *N*-glycans have two core GlcNAcs; F at the start of the abbreviation indicates a core-fucose α1,6-linked to the inner GlcNAc; Mx, number of mannose on core GlcNAcs; Ax, number of antenna (GlcNAc) on trimannosyl core; A2, biantennary with both GlcNAcs as β1,2-linked; A3, triantennary with a GlcNAc linked β1,2 to both mannose and the third GlcNAc linked β1,4 to the α1,3 linked mannose; B, bisecting GlcNAc linked β1,4 to β1,3 mannose; Gx, number of β1,4 linked galactose on antenna; Fx, number of fucose linked α1,3 to antenna GlcNAc; Sx, number of sialic acids linked to galactose.

Whole urine *N*-glycome in endometriosis



(B)

GP	Structure
GP 1	A1
GP 2	M4
GP 3	_
GP 4	FA1
	A2
GP 5	A1G1
	FA1GalNAc[SO4-2]1
	FM4
GP 6	M4A1
GP 7	A2B
GP 8	FA2
GP 9	A2GalNAc2[SO4-2]2
	M5
	FA2B
	A2G1
GP 10	FA1G1
GP 11	A2B[6]G1
	M4A1G1
GP 12	FA2G1
GP 13	A2B[3]G1
	A1GalNAc1S(6)1
GP 14	FA2BG1
	A2G2
	FA2G1GalNAc[SO4-2]1
	FA2GalNAc2
	A1G1Sg1S(3)1
GP 15	M6 D1 or D2
	A1[6]G1S(6)1
GP 16	FA1[6]G1S(3)1
	FA1GalNAc1S(3)1
	FA3G1
	A1[3]G1S(6)1
	FM4A1G1
GP 17	A1GlcA1G2S1
	A2BG2

GP	Structure
GP 18	M5A1G1
	A2G1GalNAc1[SO4-2]1S(3)1
GP 19	M4A1G1S(3)1
	A2[6]G1S(6)1
	FA2G2
GP 20	A2G2S(3)(1Ac)1
	FA1[6]G1S(6)1
	FA2F1GalNAc2
	FA2G1S(6)1
	FA1[3]G1S(6)1
	A2[3]G1S(6)1
	FA2GalNAc1S(6)1
	M4A1G1Sg1S(6)1
	FA2BG2
GP 21	FA2G1GalNAc[SO4-2]1S(3)1
	M7
	M4A1G1S(6)1
	A2G1GalNAc[SO4-2]1S(6)1
	A2G2S(3)1
	A2B[3]G1S(6)1
	A2G1G[SO4-2]1S(3)1
	FA3G2
GP 22	FA2GalNAc2S(6)1
	FA2BG1GalNAc[SO4-2]1S(3)1
	FA2F1G2
	FA2G1GalNAc[SO4-2]1S(6)1
GP 23	FA2G1G[SO4-2]1S(3)1
	M5A2BG1
	FA2G2S[3]1
	FA2BG1GalNAc[SO4-2]1S(6)1
	FA2G1GalNAc[SO4-2]1S1
	A2G2S(6)1
	FM4A1G1Sg1S(6)1
	A3BG3

GP	Structure
GP 24	A2G1GalNAc1S(3,6)2
	FA1A1[SO4-2]1G2Ga1S(3)1
	A2BG2S(6)1
GP 25	FA4G3
26	FA2G2S(6)1
	M8
	M5A1G1S(6)1
GP 27	A2G2S(6)1S(3)(Ac)1
	M5A2G2Ga2
	FM5A2G2Ga1S[6]1
GP 28	FA4G3
	FA2F1G2(Ac)2Ga1(Ac)1
	FA2G1G1[SO4-2]1S(6)1
	FA2BG2S(6)1
	A3G2G[SO4-2]1S(3)1
GP 29	FA2G2S(3)2
	FA2G1GalNAc1S(3)2
	FA2BG2S(3)2
	FA2GalNAc2S(6)2
GP 30	FA2F1G2(Ac)2Ga1(Ac)1
	FA3G3S(3)1
	M9
	A3G2G[SO4-2]1S(6)1
GP 31	A2G2S(6)2
	FA2G1GalNAc1S(6)2
	FA2G2S(3,6)2
GP 32	FA2F1G2(Ac)2Ga1(Ac)1
	FA3G2S(3)2
	FA3G3S(3)1
	A2BG2S(6)2
	FA2BG2S(3,6)2
	A3G3S(3)2
	FA2G1G[SO4-2]1S(6)2
	FA4F1G3S(3)1

GP	Structure
GP 33	FA2G2S(6)2
GP 34	FA3G3S[6]1
GP 35	FA2BG2S(6)2
GP 36	FA3G3S(3)2
GP 37	A3G3S(6)2
	FA1A[SO4-2]1G2S(6)2
GP 38	FA3BF2G3S(3)1
	FA3G3S(6)2
GP 39	A3G3S(6)3
GP 40	FA3BG3S(3)2
	FA3G3S(3)3
GP 41	A3G3S(6)3
	FA3BG3S(6)3
	FA3G3S(6)3
GP 42	A3G3S(6)3
GP 43	FA3BG3S(6)3
	FA4G3GalNAc1S4
	A3G3S(3)4
GP 44	FA3F1G3S(3)3
	FA4BG3S(3,6,6)3
	FA4G4S(3)3
GP 45	FA3F1G3S(6)3
	A3G3S(6)4
	A4G4S(3)4
GP 46	FA4G4S(6)3
GP 47	_
GP 48	A4G4S(6)4
GP 49	FA4F1G4S(3)4
GP 50	FA3GalNAc1G2S(6)3

Figure 3. (A) Representative HILIC-UPLC chromatogram of the *N*-glycans from all urine glycoproteins of a control (without endometriosis) sample and highlighted GP36 which is the only significantly altered GP in endometriosis. **(B)** Glycan structures in each GP of the whole urine *N*-glycome profile.

Conclusion

The 2AB labelled whole serum profile is the most appropriate potential source of non-invasive biomarkers.

ProA labelled serum IgG also shows differences and potential biomarkers, and as an immunological glycoprotein, it could be a good target for potential diagnosis and treatment.

A novel method was developed for extraction of *N*-glycans from non-invasively collected urine samples.

Total urine glycoproteins were analyzed for detailed *N*-glycan composition for the first time.

Discussion

The statistical analysis of our samples indicates that many GPs are significantly altered in 2AB labelled whole serum samples even if the MFC cohort includes both luteal and proliferative samples, and hormones do affect glycosylation. Therefore, sampling consistency is crutial and further investigation has to be done with a bigger sample cohort and samples should be further collected from the same hormonal phase.
IgG *N*-glycosylation was analyzed from all serum and urine samples using ProA labelling as IgG is a low abundant protein of urine and therefore, a new method was required for glycan analysis. The 4-amino-N-[2-(diethylamino)ethyl] benzamide (ProA) operates with the same mechanism as 2AB to bind to the reducing end of a glycan using Schiff base chemistry. ProA shows increased fluorescence and ionization performance, which can be explained by the fact that it contains a basic tertiary amine tail, hence it provides higher sensitivity during mass spectrometry and liquid chromatography separation.
A novel method was developed for the analyses of the *N*-glycans from whole urine glycoproteins and IgG, which was able to overcome the limitations of urine, to allow effective, reproducible analysis.

References

(1) Jasim Rasha Z, et al. Sialic acid is a novel biochemical marker in sera of Iraqi endometriotic patients. J Nat Sci Res 2014, 4(10).
(2) Royle L, et al. HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. Anal. Biochem. 2008, 376 (1), 1−12.
(3) Royle L, et al. Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions. Methods Mol Biol 2006, 347, 125–143.
(4) Kovacs Z, et al. Procainamide fluorescent labeling for identification of low abundant non-invasive glycan biomarkers, in preparation.
(5) Saldova R, et al. Association of N-glycosylation with breast carcinoma and systemic features using high-resolution quantitative UPLC. J Proteome Res. 2014; 13(5):2314-27.
(6) Pučić M, et al. High throughput isolation and glycosylation analysis of IgG-variability and heritability of the IgG glycome in three isolated human populations. Mol Cell Proteomics. 2011,10(10):M111.010090.

Disclosures

The Protein G tips (Hamilton Microlab STARlet system, 1000 ul tip, 20 ul bed-volume, PTH 91-20-02 Box of 96 **PhyTip columns** (1 mL volume) 20 µL of Protein G affinity resin per column) used for the IgG capture, both from serum and urine, was a gift from **Biotage, PhyNexus**, (Uppsala, Sweden).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 843862, H2020-MSCA-IF-2018.