SAŽETAK

Rak dojke trenutno je najčešće dijagnosticirani tip raka kod žena. Trenutne metode nedovoljno dobro prate i predviđaju reakcije pacijenata na liječenje antihormonskim lijekovima, izlažući pacijente potencijalno nepoželjnim nuspojavama tih lijekova. N-glikani plazmatskih proteina i imunoglobulina G (IgG) pokazani su kao potencijalni biljezi mnogobrojnih bolesti, posebno onih s izraženim upalnim ili metaboličkim komponentama poput dijabetesa, hipertenzije, kardiovaskularnih bolesti i karcinoma. Anastrozol i tamoksifen su često primjenjivani lijekovi u liječenju Luminal A i Luminal B podtipova raka dojke, gdje hormonski faktori, poznatog utjecaja na IgG glikozilaciju, igraju značajnu ulogu u napredovanju raka. Za analizu primijenjen je automatizirani protokol koji je uključivao izolaciju IgG, denaturaciju proteina, fluorescentno obilježavanje glikana, pročišćavanje i konačnu analizu pomoću CGE-LIF. Rezultati studije otkrivaju različite trendove u promjenama glikana uzrokovanih terapijama anastrozolom i tamoksifenom. Terapija anastrozolom rezultirala je marginalnim, ali dosljednim pozitivnim promjenama u agalaktozilaciji (G0), račvajućem N-acetilglukozaminu (B) i sržnoj fukozilaciji (CF), dok su monogalaktozilacija (G1), digalaktozilacija (G2) i sijalinizacija (S) pokazivali smanjenje trendove tijekom vremena. Međutim, nakon primjene statističke korekcije, nestala je značajnost promjena u G0 i S. Nasuprot tome, ispitivanje tamoksifena, otkrilo je suprotne trendove u usporedbi s anastrozolom, pri čemu su svi glikanski atributi, osim B i G2, pokazali različite trendove. Konkretno, G0, G2 i CF su se smanjivali, dok su G1, S i B povećavali tijekom vremena. Usporedba učinaka terapija tamoksifenom i anastrozolom na glikanske karakteristike istaknula je različite obrasce. Dok je G0 pokazivao smanjenje s tamoksifenom u usporedbi s anastrozolom, ostali atributi galaktozilacije, G1 i G2, pokazali su neznačajne razlike između dvije terapije, s pozitivnim vremenskim efektima od 0.01751 i 0.02465. Značajno, atribut S je pokazao značajan porast (vremenski efekt 0.10811) s tamoksifenom u usporedbi s anastrozolom, ukazujući na različite utjecaje na sijalinizacijske obrasce. Suprotno tome, iako atribut B nije pokazao značajne promjene, CF je pokazao značajan pad (vremenski efekt -0.09636) s tamoksifenom u usporedbi s anastrozolom, ističući različite učinke na CF koji su ostali značajni čak i nakon korekcije. Ovo je prvo takvo istraživanje koje uspoređuje glikozilacijske obrasce u antihormonskoj terapiji raka dojke, te ovdje opisani rezultati daju temelj za daljnje glikomske studije raka dojke.

Ključne riječi: *N*-glikozilacija, *N*-glikani, rak dojke, *N*-glikani imunoglobulina G, automatizacija laboratorija, UHPLC

SUMMARY

Introduction

Protein glycosylation, a well-regulated and evolutionarily conserved co- and post-translational modification, involves the covalent attachment of glycans. Depending on the type of linkage between oligosaccharides and proteins, N-, O-, C-, S-, and P- (phosphoserine glycosylation) are distinguished. It's estimated that at least half of all human proteins are glycosylated. Glycans exist either in free form or attached to proteins, lipids, and nucleic acids, influencing the structure and function of proteins, and playing crucial roles in nearly all physiological processes. N-glycosylation, where the glycan attaches to the protein via the nitrogen in the side chain of asparagine, is the most prevalent and extensively studied form of glycosylation.

Immunoglobulin G (IgG), a simple glycoprotein found in human plasma, contains two conserved N-glycosylation sites in the Fc region, while 15–25% of plasma IgG has attached glycans in the variable Fab regions. The glycosylation of IgG has been well studied, and changes in glycosylation can affect the protein's function. IgG serves as a key mediator of the immune response by binding to Fc gamma receptors on immune effector cells and by activating the C1q component of the complement, leading to complement-dependent cytotoxicity. Glycosylation determines whether IgG molecules will have pro-inflammatory or anti-inflammatory effects. Galactosylated or sialylated IgG molecules are frequently linked to anti-inflammatory effects, while their non-galactosylated or non-sialylated counterparts tend to be associated with pro-inflammatory effects. In addition, glycans lacking core fucose have been reported to display pro-inflammatory effects.

The glycosylation of IgG changes based on physiological conditions, such as ageing, sex hormone influence, changes in lifestyle factors, and pathophysiological states, including rheumatoid arthritis, diabetes, cancers, cardiovascular diseases, and infectious diseases. Glycans are highly stable within individuals (intraindividual stability) and observed changes in pathological conditions make them potential candidates as diagnostic and prognostic biomarkers. Due to the complex structure and similar properties among glycans, the analysis and separation of individual glycan structures has historically been challenging and labour-intensive. Automating certain steps can significantly increase the number of samples processed per day, improve analysis reproducibility, reduce potential errors, and mitigate risks for personnel working with hazardous samples. Sample preparation is often the most demanding

and time-consuming step in glycomics analysis, and automating this step would accelerate the analysis significantly.

Breast cancer (BC) is the most common type of cancer among women. Around 80% of breast tumours are oestrogen receptor (ER)-positive. ER-positive breast tumours can be treated with antihormonal therapy, but resistance can develop. Postoperative antihormonal therapy involves various treatment strategies, including ovarian function suppression, aromatase inhibitors (AIs), selective oestrogen receptor modulators (SERMs), and selective oestrogen receptor downregulators (SERDs). Oestrogen plays a complex role in regulating inflammatory processes, primarily through oestrogen receptors (ERα and ERβ) on immune cells and through direct effects on the cell nucleus. Changes in oestrogen and progesterone levels can influence disease activity.

The literature on IgG glycosylation in BC is scarce, but significant differences in Fc IgG glycosylation between women with BC and healthy controls have been demonstrated, and specific glycan structures have been proposed as potential biomarkers for early detection of BC. There are no studies that have tracked changes in IgG glycosylation as a response to antihormonal therapy in BC patients. Given the established influence of BC and oestrogen on IgG glycosylation, it can be expected that such therapy will impact IgG glycosylation.

Materials and methods

This study focused on adults recently diagnosed with breast cancer, utilizing MRI, ultrasound, and mammography alongside core biopsies to determine the disease's clinical stage (cT1-4N+) and biological characteristics. Participants with Luminal A and Luminal B types, excluding those with metastatic disease or undergoing neoadjuvant therapy or GnRH agonists, were included. Menopausal status was assessed for the group receiving anastrozole treatment. Samples were collected at the Cancer Clinic of Sisters of Charity University Hospital Centre in Zagreb, Croatia, with patients' IgG N-glycosylation profiles analysed. The research received approval from the Ethics Committees of the Sisters of Charity University Hospital Centre and the Faculty of Pharmacy and Biochemistry and adhered to Helsinki Declaration principles. Blood plasma samples, collected over 9 months at 3-month intervals during various treatment stages, were stored at -80°C. Each sample was coded to protect participant identities, containing organized data covering general information, pathohistological details of cancer, administered therapies, and collection specifics.

IgG isolation was initiated by applying 25 µL of plasma on a CIM® r-Protein G LLD 0.05 mL Monolithic 96-well Plate. The isolation step was followed by an automated step of glycan deglycosylation by SDS and incubation at 60 °C. Glycan release was then achieved enzymatically by overnight treatment with PNGaseF at 37 °C. Released N-glycans were then fluorescently labelled with 8-aminopyrene-1,3,6-trisulfonic acid using a reductive amination reaction with 2-picoline borane as a reducing agent. The automated preparation was finished off by separating glycans from the matrix using hydrophilic interaction liquid chromatography solid-phase extraction on wwPTFE filter plates filled with Bio-Gel P-10. Purified glycans were eluted with ultrapure water and then analysed using a 3500 Genetic Analyzer for capillary gel electrophoresis with laser induced fluorescence (CGE-LIF) analysis. The resulting electropherograms were integrated manually into 27 glycan peaks using Empower 3 software, and the glycan structures in each peak were calculated and subjected to statistical analysis. From these peaks, 6 derived glycan traits with common structural features, such as galactosylation or sialylation, were calculated.

The CGE data for glycan underwent standardization, including total area normalization, log transformation, and batch correction using the ComBat method in R software. Glycan peak values were then reverted to their original scale for deriving traits. Glycan trait values were transformed using inverse rank transformation for normality. Time's effect on derived glycan traits was analysed using a linear mixed model with p-values adjusted via the Benjamini–Hochberg method.

Results

In our study, we analysed the impact of breast cancer therapies on glycan traits, focusing initially on anastrozole, an AI. Notably, anastrozole treatment showed marginal yet consistent positive changes in G0 (time effect: 0.04639), B (time effect: 0.01696), and CF (time effect: 0.02219), while G1 (time effect: -0.00395), G2 (time effect: -0.03684), and S (time effect: -0.05190) exhibited time dependent decreasing trends. However, after applying statistical correction, the significance of changes in G0 and S was no longer maintained. Conversely, examination of tamoxifen, a SERM drug, revealed opposing trends compared to anastrozole, with all glycan traits, except for B and G2, showing different trends. Specifically, G0 (time effect: -0.02304), G2 (time effect: -0.00408), and CF (time effect: -0.05364) decreased, while G1 (time effect: 0.01694), S (time effect: 0.0794), and B (time effect: 0.04254) increased over

time. Although initial analysis showed significant time effects for S and CF with tamoxifen, these traits became insignificant after correction.

Comparing the effects of tamoxifen and anastrozole therapies on glycan traits highlighted distinct patterns. While G0 exhibited a decrease (time effect of -0.05118) with tamoxifen compared to anastrozole, other galactosylation-related traits, G1 and G2, displayed insignificant differences between the two therapies, with positive time effects of 0.01751 and 0.02465, respectively. Notably, the S trait exhibited a substantial increase (time effect of 0.10811) with tamoxifen compared to anastrozole, indicating differential impacts on sialylation patterns. Conversely, although the B trait showed no significant changes (time effect of 0.03973), CF demonstrated a marked decrease (time effect of -0.09636) with tamoxifen compared to anastrozole, underscoring distinct effects on CF that remained significant even after correction.

Conclusions

This study aimed to unravel the complex interplay between glycan alterations and therapeutic interventions in breast cancer (BC), particularly focusing on the effects of two widely used medications: anastrozole, an AI, and tamoxifen, a SERM. These drugs are pivotal in treating Luminal A and Luminal B subtypes of BC, where hormonal factors play a significant role in cancer progression. Glycan alterations are increasingly recognized as crucial players in BC progression, extending beyond mere changes in overall serum protein glycosylation. Previous research has identified specific glycan structures, such as FA2, as potential biomarkers for early BC detection. These findings underscore the importance of investigating glycosylation patterns during BC therapy and understanding how patients respond to these treatments. Moreover, recent studies have shed light on the intricate relationship between oestrogen and IgG Fc glycosylation, revealing oestrogen's direct regulatory role in modulating IgG Fc galactosylation and sialylation. This connection between hormonal status and immune system modulation adds another layer of complexity to the glycan alterations observed in BC patients undergoing hormone-targeted therapies. The study findings reveal distinct trends in glycan alterations induced by anastrozole and tamoxifen therapies. Anastrozole therapy resulted in reduced levels of digalactosylation and sialylation, potentially attributed to its broad suppression of oestrogen levels throughout the body. Conversely, tamoxifen therapy showed a tendency towards reducing agalactosylation and increasing sialylation, reflecting its selective action on oestrogen receptors in cancer cells. Interestingly, individual responses to therapy

varied significantly, with notable outliers in glycan alterations observed among patients. These diverse responses underscore the complexity of individual reactions to therapy, suggesting the need for personalized treatment approaches in BC management. Despite the valuable insights provided by this study, several limitations should be acknowledged. The relatively short follow-up duration, spanning a maximum of 9 months, may not fully capture long-term treatment effects. Additionally, the absence of control groups limits the depth of understanding of observed changes compared to baseline or untreated conditions.

Looking ahead, future investigations should extend the sampling period to encompass long-term treatment effects and recruit more individuals for more robust and increase the power of their studies. By addressing these limitations, future research can provide a more comprehensive understanding of glycan alterations in BC therapy and pave the way for personalized treatment strategies tailored to individual patient needs.

Keywords *N*-glycosylation, *N*-glycans, breast cancer, Immunoglobulin G *N*-glycans, laboratory automation, CGE-LIF