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Research Article

DECIPHERING THE IMPACT OF COLLAGENASE TREATMENT DURATION ON PRIMARY BREAST CANCER CELL PROTEOME: A COMPREHENSIVE STUDY

Merve Gülsen BAL ALBAYRAK [1], Murat KASAP [1], Gürler AKPINAR [1], Sevinç YANAR [2], Turgay ŞİMŞEK [3], Nuh Zafer CANTÜRK [3]

[1] Department of Medical Biology, Medical School, Kocaeli University, 41001 Kocaeli, TURKIYE
[2] Department of Histology and Embryology, Medical School, Sakarya University, Sakarya, TURKIYE
[3] Department of General Surgery, Medical School, Kocaeli University, 41001 Kocaeli, TURKIYE
*Correspondence: mervegulsenbal@gmail.com

ABSTRACT

Objective: Primary cell isolation is essential for studying cellular behavior and disease mechanisms, with collagenase-mediated tissue dissociation playing a critical role in the process. However, the impact of collagenase treatment duration on the proteome of primary cells, particularly in breast cancer research, remains underexplored. This study aims to investigate the effects of collagenase II treatment duration on the proteomic profiles of primary breast cancer cells.

Materials and Methods: Breast cancer tissues from patients diagnosed with infiltrating ductal carcinoma were treated with collagenase II for either 1 or 3 hours. Subsequent proteomic analysis was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Identified proteins were subjected to bioinformatic analyses to determine the functional implications of the proteomic changes induced by the different treatment durations.

Results: Bioinformatic analyses showed that 1-hour treatment predominantly affected proteins involved in cytoskeletal organization and cell adhesion, with significant enrichment in actin cytoskeleton dynamics and structural molecule activity. In contrast, 3-hour treatment led to significant metabolic reprogramming, with enhanced regulation of pathways involved in energy production, including the TCA cycle and glycolysis.

Conclusion: This study reveals for the first time that, collagenase II treatment duration significantly alters the proteomic profile of primary breast cancer cells, with shorter durations affecting structural proteins and longer durations inducing metabolic changes. Optimizing treatment time is crucial for targeted proteomic studies.

Keywords: Primary cells, breast cancer, collagenase, treatment duration, proteomics

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INTRODUCTION

Cell culture studies serve as vital tools to uncover the complexity of cellular behavior and provide crucial information for understanding disease pathology and therapeutic development (1). Central to these efforts is the isolation and cultivation of primary cells that closely mimic in vivo cellular physiology when compared to immortalized cell lines (2). Among primary cell isolation techniques, enzymatic tissue dissociation using collagenase is especially important (3).

Collagenase, an enzyme that cleaves peptide bonds within collagen fibers, plays an important role in tissue dissociation and facilitates the release of individual cells from complex tissue matrices (3). Collagen, a key component of the extracellular matrix (ECM), provides structural support to tissues and serves as a scaffold for cell adhesion and migration. Collagenase specifically degrades collagen in the ECM, allowing for the release of cells embedded within the tissue matrix (4). Collagenase exerts its enzymatic activity by cleaving specific peptide bonds within the triple helical structure of collagen molecules. Specifically, collagenase targets the peptide bonds between glycine and other amino acids in the collagen polypeptide chain, leading to the degradation of collagen fibers into smaller fragments. This enzymatic degradation softens the tissue matrix, facilitating the dissociation of cells from the surrounding ECM (5).

Several types of collagenases, derived from bacterial and mammalian sources, are used in primary cell isolation protocols. Among the most used are Collagenase I, Collagenase II, and Collagenase IV, each exhibiting distinct substrate specificities and enzymatic properties (6,7). Collagenase I (also known as Clostridium histolyticum collagenase) preferentially cleaves native collagen fibrils, making it suitable for the isolation of primary cells from tissues rich in type I collagen, such as skin and bone. Collagenase II (derived from Clostridium histolyticum) exhibits broader substrate specificity, allowing for the efficient dissociation of various tissue types, including cancer tissues abundant in both type I and type III collagens (8). Collagenase IV, sourced from Vibrio proteolyticus, demonstrates high specificity for denatured collagen, making it suitable for the isolation of cells from decellularized tissues and cell culture applications (9).

Numerous studies have explored the use of collagenases in primary cell isolation, assessing its effectiveness across different experimental conditions and tissue types (10–12). These studies used collagenase at various concentrations, incubation times, and temperatures, reflecting the different approaches researchers have taken to optimize tissue dissociation protocols. While some studies advocate long-term collagenase treatment to maximize cell yield and viability, others recommend shorter incubation periods to minimize potential changes in cellular phenotype and function (13,14). Furthermore, inconsistencies exist regarding the effect of collagenase treatment duration on downstream assays, including proteomic profiling, and conflicting reports highlight the need for further research.

Despite the wealth of literature on collagenase-mediated primary cell isolation, a notable gap exists in our understanding of the relationship between collagenase treatment duration and the resulting proteome. In breast cancer research, there is a notable lack of studies systematically investigating the effects of varying collagenase treatment durations on the proteomic composition of primary cells (15). This lack of understanding presents a major challenge for researchers working to unravel the molecular complexities of breast cancer progression and to create targeted therapeutic strategies.

In light of these considerations, the current study aimed to address this issue by comprehensively investigating how collagenase treatment duration affects the proteome of primary breast cancer cells. Through sensitive experiments and comprehensive LC-MS/MS analysis, we aimed to reveal the molecular effects of collagenase-mediated tissue dissociation and provide valuable information to optimize primary cell isolation protocols for subsequent proteomic studies. By unraveling the complex relationship between collagenase treatment duration and the primary cell proteome, our goal is to improve the reproducibility and reliability of experimental results, thereby advancing our understanding of breast cancer biology and aiding the development of innovative therapeutic approaches.

MATERIALS AND METHODS

Patient Characteristics

Breast cancer tissues were collected under ethical approval number KU GOKAEK-2019/16.04 2019/139 by Ethics Committee of Kocaeli University. Patients that were diagnosed as infiltrating ductal carcinoma (IDC) by the General Surgery Clinic of Kocaeli University School of Medicine between years 2019 and 2021 were chosen. Patients were selected among the ones that were not received any drug therapy. After removal, the tissues were washed with proteomic wash buffer (10 mM Tris buffer containing 250 mM sucrose at pH 7.4), and then immediately taken to the collagenase treatment procedure within 30 minutes.

Cell Culture and Collegenase II treatment

The tissues were diced into small pieces and washed with wash buffer to remove excess blood. After centrifugation at 10000×g, 500 µl of DMEM (Thermo Fisher Scientific, USA) medium containing 300 U/ml Collagenase II was added to the tissue pellets. Two groups were established for collagenase treatment durations of 1 hour and 3 hours. The tubes containing the tissue and medium-collagenase mixture were incubated at 37°C for either 1 hour or 3 hours, depending on the group. Following incubation, the tubes were centrifuged at 7000×g at 4°C for 10 minutes. Supernatants were removed, and EASYpack Protease Inhibitor Cocktail (cOmplete™ ULTRA Tablets, Merck, Darmstadt, Germany) was added to the pellets. Protein isolation experiments were then carried out using these samples.

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Protein Isolation

Stainless steel beads (1.4 mm) were added to the tubes containing tissues treated with collagenase II. The tissues were then homogenized using a bead-beater (Bullet Blender, Next Advance, Troy, NY, USA) at speed 9 for 4 minutes, repeated five times. The homogenates were centrifuged for 10 minutes at 10,000×g at 4°C, and the supernatant was transferred into fresh tubes. These tubes were centrifuged again for 45 minutes at 18,000×g at 4°C to obtain a clear crude extract, and the supernatants were transferred into fresh tubes and labeled. Protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA, USA) with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The protein extracts were briefly immersed in liquid nitrogen for approximately 30 seconds to rapidly freeze them, and then transferred to storage at -80°C for further use.

Preperation of Protein Pools and In Solution Tryptic Digestion

To prevent sample-to-sample variation, protein pools were created by mixing equal amounts of protein from each patient's sample into a single tube. Before LC-MS/MS experiments, the protein pools were cleaned to remove impurities using TCA/acetone precipitation. Briefly, 200 µg of protein from each pool was diluted to 90 µl with distilled water, and 10 µl of 0.2% DOC (deoxycholate.Na) solution was added. The samples were incubated on ice for 15 minutes. Next, 10 µl of 72% TCA (trichloroacetic acid) was added, and the samples were incubated on ice for another 15 minutes. Following centrifugation at 12,000×g for 10 minutes at 4°C, the supernatants were removed, and 1 ml of ice-cold acetone was added. The tubes were mixed well with a vortex and incubated at -20°C for 30 minutes. After incubation, the tubes were centrifuged again at 12,000×g for 10 minutes at 4°C. The supernatants were removed, and the protein pellets were air-dried and dissolved in 50 mM ammonium bicarbonate. Protein concentrations of the pooled samples were determined using the Qubit Protein Assay (Thermo Fisher Scientific, USA) before further experiments.

Prior to LC-MS/MS analysis, proteins were digested into peptides using in-solution tryptic digestion and guanidination kit (Thermo Scientific, USA) as described in our previous work (16).

Liquid chromatography (LC)-tandem MS/MS analysis

The digested peptides were analyzed by nLC-MS/MS, following the method outlined by Yanar et al. (17) Data-dependent acquisition was utilized, targeting the top 10 precursor ions for MS/MS analysis, with an MS scan mass range of 400–2000 m/z. Raw data were worked using Proteome Discoverer SEQUEST (version 2.2; Thermo Fisher Scientific) according to the parameters specified in our previous work (17). Protein identification was performed against the Uniprot/Swissprot database.

Bioinformatic analysis

Protein-protein interactions, biological functions, and molecular pathways linked to identified proteins were explored using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, as previously described (17). STRING (https://string-db.org) was employed with previous parameters and interaction sources(17). Results showing significant findings with a false discovery rate (FDR) below 1e-05 were visualized and analyzed using Adobe Illustrator Version 6. Enrichment analyses included GO terms (biological processes (BP), cellular components (CC), molecular functions (MF)), KEGG pathways, Reactome (RKTM), and WikiPathways (WP) with a p-value < 0.05. Bubble plots showing the enriched results were created using SRPlot.

RESULTS

Comparative proteome analysis of primary cells

The effects of collagenase II treatment duration on cells were assessed by examining alterations in global protein expression profiles. The digested proteins from each group were analyzed using nHPLC coupled with LC-MS/MS. Proteomic analysis identified 272 proteins in cells treated with collagenase II for 1 hour, while 259 proteins were identified in cells treated for 3 hours. After filtering for proteins identified by at least two unique peptides, the number of identified proteins decreased to 127 for the 1-hour group and 118 for the 3-hour group. A comparison of the identified proteins between the two groups revealed 162 common proteins, as illustrated in the Venn diagram (Figure 1). Additionally, 110 proteins were unique to the 1-hour treatment group, and 97 proteins were unique to the 3-hours treatment group.

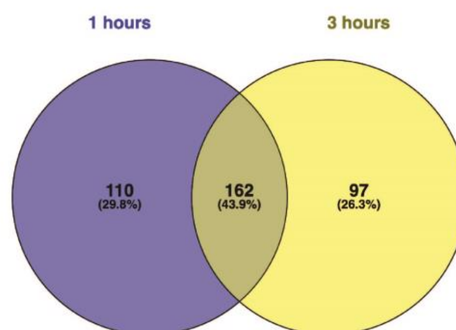

Figure 1. Venn diagram of LC-MS/MS identified proteins for the 1-hour and 3-hour collagenase treatment groups. The numbers represent the gene counts for the corresponding groups.

Bioinformatic analysis of identified proteins

To link the identified proteins with protein networks and molecular pathways, GO terms related to Biological Process (BP), Cellular Component (CC), Molecular Function (MF), along with KEGG pathways,

MEANDROS
MEDICAL AND DENTAL JOURNAL
September 2024 (3):222-232

Reactome, and WikiPathways, were analyzed using the STRING database. The outcomes from analyses were integrated and visualized as bubble plots generated with SRPlot (Figure 2 and Figure 3).

For cells treated for 1 hour, GO analysis indicated significant regulation of 'cytoskeleton organization' (Biological Process) and 'structural molecule activity' (Molecular Function). KEGG pathway analysis identified the most enriched pathways as "Regulation of actin cytoskeleton" and "citrate cycle (TCA cycle)." WP analysis highlighted key terms, with a notable entry being "metabolic reprogramming in colon and pancreatic cancer." RKTM analysis uncovered significant pathways, including "Axon guidance" and "signaling by Rho GTPases" (see Figure 2a and b).

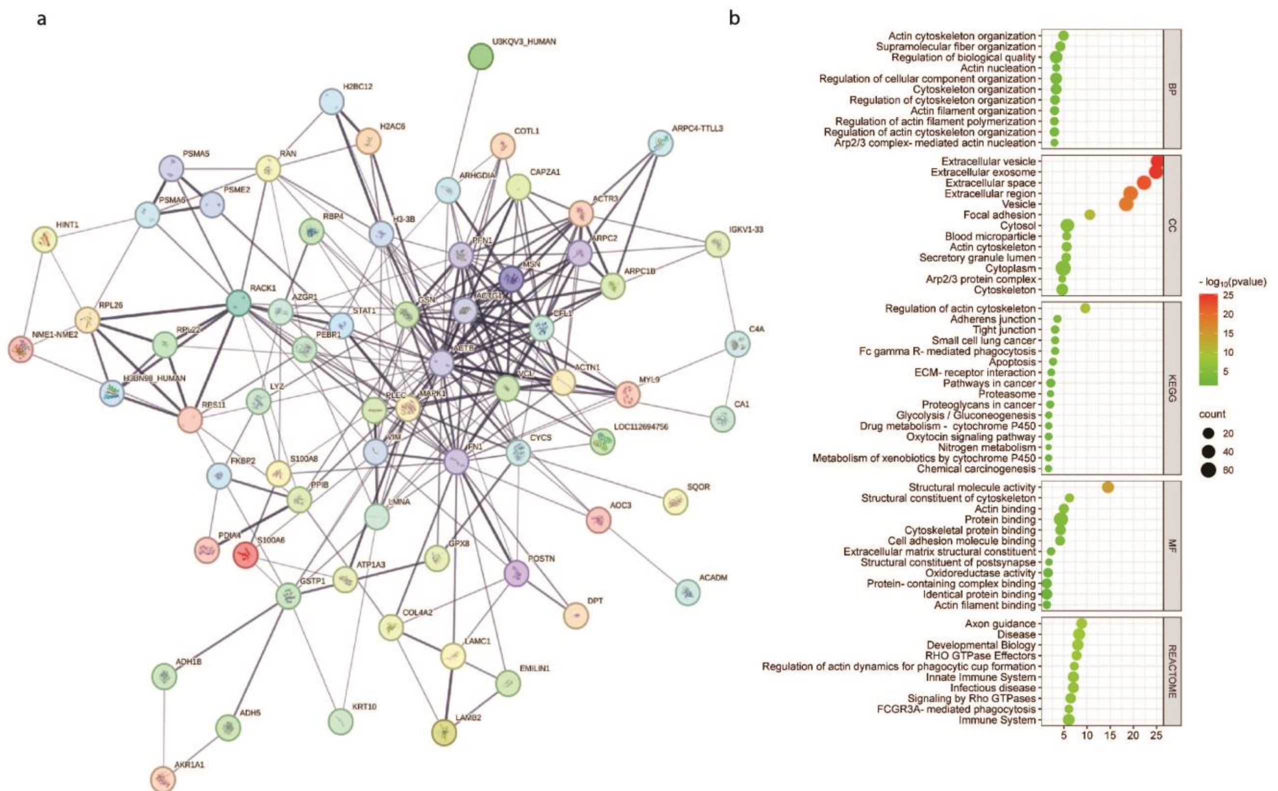

Figure 2. Pathway enrichment analyses of differentially regulated proteins identified through LC-MS/MS. a. show the STRING network analyses for the 1-hour. b. illustrate the enrichment analysis results using bubble plots for the 1-hour treatment group, based on data from Gene Ontology (GO) Biological Process (BP), Cellular Component (CC), Molecular Function (MF), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome (RKTM)

For cells treated for 3 hours, GO analysis identified important terms, with notable regulations in "Generation of precursor metabolites and energy" and "aerobic respiration" (biological process category) and "RNA binding" (molecular function category). KEGG pathway analysis revealed the enriched pathway "carbon metabolism." WP analysis highlighted significant mechanisms including "TCA cycle" and "Metabolic reprogramming." RKTM analysis showed crucial regulated pathways, such as "Citric acid cycle" and "metabolism" (see Figure 3a and b). Overall, bioinformatic analyses revealed that in cells treated for 1 hour,

the key regulatory trends were associated with actin cytoskeleton dynamics, structural organization, cell adhesion, and related signaling pathways. Conversely, in cells treated for 3 hours, the main regulatory activities were linked to metabolic processes, including energy generation, ATP metabolism, carbon metabolism, and the TCA cycle. Further analysis showed variations in expression levels among the proteins common to both treatment durations. This indicates that while some core proteins remain consistent across different treatment times, their functional roles and interactions may be modulated by the extent of collagenase exposure.

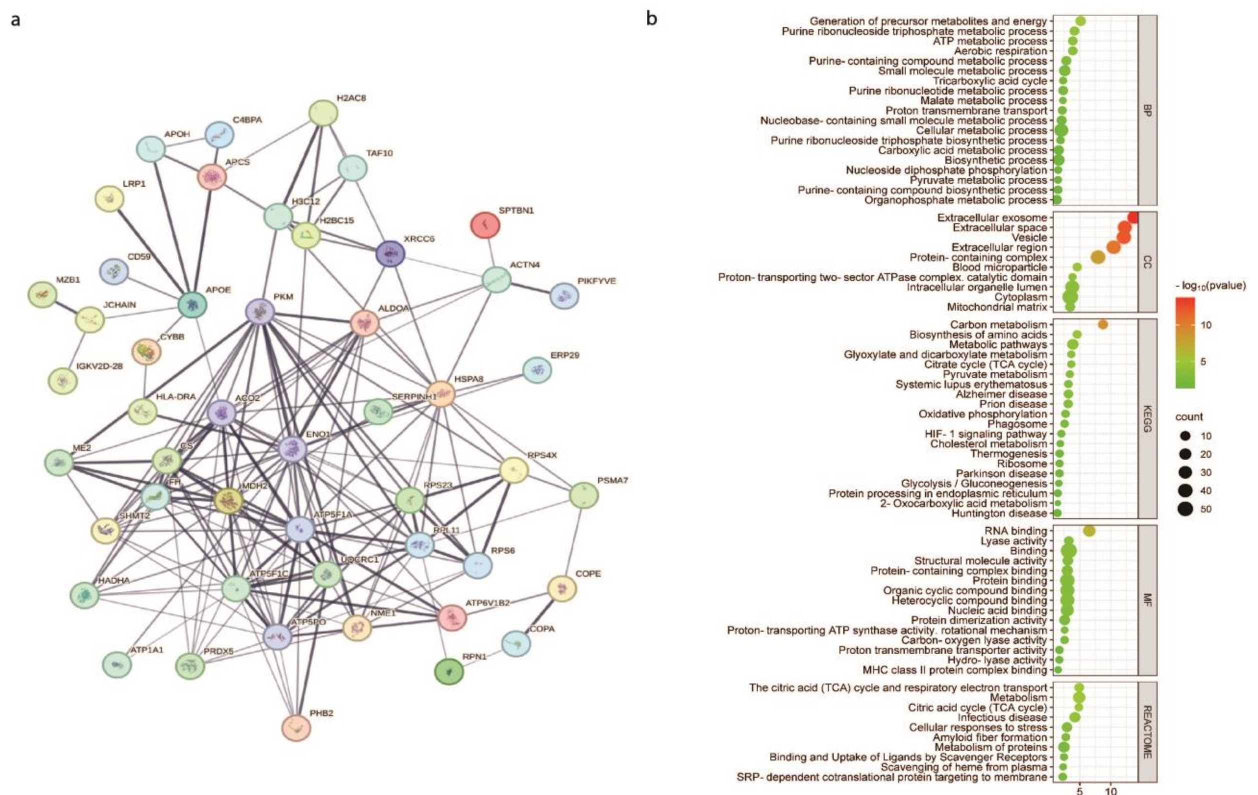

Figure 3. Pathway enrichment analyses of differentially expressed proteins, identified via LC-MS/MS, a. demonstrate STRING network analyses corresponding to the 3-hour treatment group. b. Enrichment analysis outcomes are visualized through a bubble plot for the 3-hour treatment, utilizing datasets derived from Gene Ontology (GO) terms across Biological Processes (BP), Cellular Components (CC), Molecular Functions (MF), along with the Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome (RKTM) databases.

DISCUSSION

The study aimed to investigate the impact of different collagenase II treatment durations on the proteome of primary breast cancer cells. Bioinformatic analyses were conducted to explore the functional implications of the identified proteins. Our findings reveal significant insights into the influence of enzyme treatment duration on cellular protein expression and subsequent biological processes.

MEANDROS MEDICAL AND DENTAL JOURNAL
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Collagenase is widely used for the isolation of primary cells from various tissues due to its ability to degrade collagen in the extracellular matrix (ECM). This enzymatic activity facilitates the release of cells from tissues, which can then be cultured and analyzed. Different types of collagenases (e.g., types I, II, and IV) are used depending on the tissue type and the specific requirements of the study (3,4). Collagenase II, in particular, is frequently used for tissues rich in collagen type II, such as cartilage and some tumors. The effectiveness of the enzyme and the viability of the cells can be affected by factors such as enzyme concentration and incubation time (18,19).

Our study revealed distinct protein expression profiles between the two treatment periods. Bioinformatic analysis of the regulated proteins identified in 1-hour treated cells revealed significant regulatory patterns related to cytoskeletal organization, structural molecule activity, and cell adhesion. Proteins involved in these pathways include actin, myosin, and various actin-binding proteins such as filamin and profilin. These proteins work together to remodel the cytoskeleton in response to external signals, allowing cells to adapt their structure and function (20). Structural molecule activity is crucial for maintaining the integrity and functionality of cellular components. Proteins such as tubulins and intermediate filaments are essential for forming the structural framework of the cell, providing mechanical support, and participating in various cellular processes, including mitosis and intracellular transport.

Cell adhesion molecules (CAMs) like cadherins and integrins play a vital role in mediating cell-cell and cell-ECM interactions, crucial for tissue formation, maintenance, and repair, and participate in signal transduction pathways that regulate cell proliferation, differentiation, and migration (21). Specifically, the regulation of actin cytoskeleton and focal adhesion pathways was prominent according to our results, indicating that shorter collagenase treatment may influence cell adhesion and cancer cell migration processes. This is consistent with the fact that shorter collagenase treatment durations tend to preserve more of the structural and signaling proteins involved in cytoskeletal integrity and cell-matrix interactions. Collagenase, by breaking down extracellular matrix (ECM) components, facilitates the release of cells while maintaining key functional proteins critical for cell attachment and movement. This aligns with studies that have used collagenase for primary cell isolation where the emphasis was on minimizing enzyme exposure to retain cellular functions linked to the cytoskeleton and adhesion properties (13,14). The differential expression of proteins involved in actin cytoskeleton regulation in the 1-hour treated cells suggests a transient response aimed at remodeling the cytoskeleton to facilitate cell detachment and survival. Proteins such as Rho GTPases, which are known to regulate actin dynamics, were significantly enriched. This aligns with the findings of other studies that have reported the role of Rho GTPases in mediating cellular responses to mechanical and enzymatic stress (22).

In comparison, cells treated for 3 hours showed a different proteomic landscape, with a significant emphasis on metabolic processes, including energy generation, ATP metabolism, and the TCA cycle. The

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enriched pathways suggest that extended collagenase treatment induces a metabolic shift, likely as an adaptive response to prolonged enzymatic exposure. This metabolic reprogramming is consistent with findings that describe how cells reprogram their metabolism to adapt to various stress conditions, including enzymatic treatment (23). The TCA cycle (Krebs cycle) and oxidative phosphorylation are central to cellular energy production. Key enzymes such as citrate synthase, isocitrate dehydrogenase, and succinate dehydrogenase are involved in these pathways, facilitating the conversion of glucose and other nutrients into ATP, the cell's primary energy currency (24). Cancer cells often undergo metabolic reprogramming to support rapid growth and survival under adverse conditions, involving increased glycolysis (Warburg effect), enhanced glutaminolysis, and altered lipid metabolism (23,24). The enhanced metabolic activity observed in the 3-hour treated group reflects significant metabolic reprogramming, possibly as a mechanism to cope with the stress induced by prolonged enzymatic exposure. This metabolic shift suggests that extended collagenase treatment prompts cells to reallocate resources towards maintaining energy balance and supporting survival under adverse conditions.

The common proteins observed in both treatment groups, despite differences in their expression levels, indicate that certain core proteins are consistently present across varying treatment durations. However, their functional roles and interactions may be influenced by the degree of collagenase exposure. This variable response to enzymatic treatment reveals the intricate balance between preserving cellular structure and adapting to metabolic changes, highlighting the necessity for customized enzymatic dissociation protocols. Overall, in terms of specific proteins and pathways, the regulation of the actin cytoskeleton pathway in the 1-hour treatment group suggests that these cells maintain better structural integrity and are potentially more suited for studies involving cell motility and morphology. The actin cytoskeleton plays a crucial role in cell shape, division, and intracellular transport, and its regulation is vital for understanding cancer cell metastasis (20). For the 3-hour treatment group, the upregulation of metabolic pathways, such as the TCA cycle and glycolysis, underscores the cells' metabolic flexibility and adaptability under prolonged enzymatic stress. These findings are significant as they suggest that extended collagenase treatment primes the cells for higher metabolic activity, which might be beneficial in studies focusing on cancer cell metabolism and drug response.

CONCLUSION

In conclusion, our study shows that varying collagenase II treatment durations significantly affect the proteomic structure of primary breast cancer cells. Shorter treatment durations primarily affect proteins involved in cytoskeletal organization and cell adhesion, highlighting the critical roles of these proteins in maintaining cellular integrity and functionality during initial enzymatic exposure. Conversely, extended collagenase treatment triggers significant metabolic reprogramming, underscoring the adaptation of cells to

prolonged stress through increased energy production pathways. These differential protein expression patterns highlight the need to optimize collagenase treatment conditions based on the specific biological processes being investigated.

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Not applicable

Authorship contributions

Study conception and experimental design were performed by MGBA, M.K. and G.A. Tissue collection with surgeries and storage were performed by T.S. and N.Z.C. Sample preparation and protein isolation were performed by M.G.B.A. and M.K. LC-MS/MS analysis were performed by M.G.B.A, M.K, and G.A. Bioinformatic analysis were performed by M.G.B.A and S.Y. The first draft of the manuscript was written by M.G.B.A. and S.Y. Writing, review, and editing the draft were performed by G.A. and M.K. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availibity statement

The datasets produced during this study can be obtained from the corresponding author upon reasonable request.

Declaration of competing interest

The authors report no conflicts of interest.

Ethics

This study has been approved by the Ethics Committee of Kocaeli University with approval number; KU GOKAEK-2019/16.04 2019/139.

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