

## Impact of Dairy and Plant-Based Milk Products on the Growth Dynamics of Triple- Negative Breast Cancer Cells in Vitro

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Triple-negative breast cancer (TNBC) is the most common malignant tumor among Arab women, accounting for 18.8% of female cancer cases in Oman. TNBC lacks estrogen and progesterone receptors, as well as the Human Epidermal Growth Factor Receptor 2 (HER2). Its growth is stimulated by Insulin-Like Growth Factor 1 (IGF-1), which is secreted by the liver and found in blood, saliva, and milk. High levels of IGF-1 are found in tumor growth. Cow's milk, a complex biological fluid, is known to increase systemic IGF-1, insulin, and estrogen signaling, which may contribute to breast cancer progression. This study investigated the effects of cow's milk, soy milk, and almond milk on TNBC cell proliferation in vitro. It was hypothesized that cow's milk would enhance cancer cell proliferation and IGF-1 levels, whereas soy and almond milk would have minimal effects. The MDA-MB-231 TNBC cell line was selected for this study. The experiment was divided into four groups: Cow's Milk A, Cow's Milk B (from two different commercial brands), and Almond milk, and Soy milk as a control. These four different types of milk were introduced into cultured cancer cells at 25  $\mu\text{L}/\text{mL}$ , 50  $\mu\text{L}/\text{mL}$ , 100  $\mu\text{L}/\text{mL}$ , and 200  $\mu\text{L}/\text{mL}$  concentrations, and incubated for 24 and 48 hours. Cell viability and proliferation were assessed using MTT assay. IGF-1 and IGF-1 receptors were measured using the ELISA technique which were compared to control samples. At 24 and 48 hours, Cow's Milk A and B gradually increased cancer cell proliferation from 50 to 200  $\mu\text{L}/\text{mL}$  concentrations. Conversely, almond and soy milk did not show an increase in cell proliferation with rising concentrations. These findings suggest that cow's milk may promote TNBC cell proliferation through IGF-1 signaling, while plant-based alternatives may have a lesser impact. Additional research is necessary to better understand the influence of IGF-1 in cow's milk on the development and progression of triple-negative breast cancer (TNBC).

**Keywords:** Cow's milk; Insulin-Like Growth Factor 1; In vitro experiment; Triple-negative breast cancer; Tumor cell lines.

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In the year 2018, 15% being the triple-negative variant out of 2.3 million women who suffered from breast cancer (BCa), meaning they do not express estrogen and progesterone receptors and Human Epidermal Growth factor receptor

(HER) amplification. <sup>1</sup> BCa accounts for 12% of all new cancer cases and represents nearly one-quarter of all cases among women. <sup>2</sup> Despite frequent awareness campaigns related to lifestyle and frequent checkups, and the overall improvement

in treatment and management, a yearly increase of 3.5% in BCa incidence was observed from the year 1999 to 2011 in the UK,<sup>3</sup> and Oman following a similar pattern of increase.<sup>4</sup> Several studies have attempted to link dietary habits with cancer, and cow's milk being an increasingly popular target in recent research.<sup>5-7</sup> It was reported in Japan from 1947-1997, a 20-fold increase in milk intake resulted in 2-fold increase in death rate from BCa.<sup>8</sup>

Milk is a complex biological fluid rich in estrogen, progesterone and growth-promoting endocrine system IGF-1. Cow's milk could potentially increase the growth of hormone sensitive cancers.<sup>9</sup> Cow's milk is commercially available in all countries and its use is highly recommended during pregnancy, childhood, infancy, old and menopausal age; milk enhances endocrine postnatal signaling system enhancing IGF-1.<sup>10</sup> Humans are mammal consuming milk from other mammals because they provide calcium and Vitamin D.<sup>11</sup> IGF-1 in cow's milk is similar in structure to human IGF-1, which means it can potentially interact with human IGF-1 receptors when ingested and the process of pasteurization, which is used to kill harmful bacteria in milk, does not destroy IGF-1. This means that IGF-1 remains active in commercially available milk.<sup>11</sup>

Milk of the cow naturally contains IGF-1, which is a part of the milk's growth-promoting properties. The levels of IGF-1 in milk can vary, but generally, IGF-1 is present in concentrations ranging from 1.0 to 83 ng/mL, which depends on cow's diet, lactation period, and whether the cow has been treated with bovine somatotropin (bST), a hormone used to increase milk.<sup>11</sup> However, the premise of our research is not the effect of hormones on cancer, it is the effect of the other components of milk on cancer, namely the IGF-1, as the triple negative variant has no estrogen or progesterone receptors, low HER2 receptor expression, however, can express IGF-1 receptors.<sup>12</sup> We explore the effects of cow, Soy and Almond milk on these triple negative breast cancer cells.

Insulin-like growth factor 1 (IGF-1) is made of a single polypeptide chain, that is like insulin and IGF-2 in its structure, though it has a greater binding attraction for the IGF receptor than IGF-2.<sup>13, 14</sup> IGF-1 is crucial in tissue development, it stimulates cell growth by proliferation and differentiation, the circulating IGF-1, secreted by

the liver and fixes to the IGF-1 receptor, which in turn triggers a signal transduction cascade that will increase the cells proliferation, and promote its survival by activating RAS and AKT pathways; which regulate the genes involved in these cellular activities. The activation of these pathways will lead to the upregulation of a protein called cyclin D1; which is considered one of the most important human oncogenes, and its binding protein CDK4, resulting in the discharge of E2F transcription factor, and expression of downstream target genes, like cyclin E, which regulates the cell progression into the S phase by binding to G1 phase cdk2.<sup>15,16</sup> Moreover, the IGF-1R activation also leads to the downregulation of the cell cycle suppressors p27kip1p57kip2 and PTEN (tumor suppressor chromosome 10).<sup>17,18</sup> The normal life cycle of human cells is regulated by extra and intracellular signals, that work together to control cell proliferation, senescence, and apoptosis. As previously mentioned, the IGF-1 system is a potent pro- survival stimulus that inhibits apoptosis or programmed cell death, which is essential for embryonic development and the elimination of cancerous and virus-infected cells. The AKT or mitogen-activated protein kinase pathway is important in apoptosis by inhibiting pro-apoptotic proteins like BAD and FKHR and stimulating anti-apoptotic factors such as NF-kappa B and MDM2, which promotes the growth of cancerous cells.<sup>19, 20</sup>

Commercial cow's milk is going to increase IGF-1 levels, which in turn is going to have a positive effect on the proliferation of Triple-Negative breast cancer cells. On the other hand, Soy and Almond milk are going to have little to no effect on the proliferation of these cancerous cells.

To assess the effect of different types of milk (commercial cow's milk, soy milk and almond milk) on IGF-1 levels and the proliferation of triple negative breast cancer cells in vitro.

## MATERIALS AND METHODS

We used MDA MB-231 triple negative cell line provided by Sultan Qaboos University, department of Department of Biomedical Sciences as a part of our collaboration with them, all the needed materials for culturing and subculturing were bought with the TRC grant and the experiment.

### Cell culturing

To culture the cell line, we used the instructions provided from the ATCC website<sup>21</sup>, the cell line is received in packaged dry ice, and stored in liquid nitrogen vapor phase below -130°C. To start the culturing process, the cells were thawed in a 37°C bath for approximately 2 minutes, followed by decontamination using 70% ethanol. A complete growth medium was made by combining ATCC-formulated Eagle's Minimum Essential Medium with fetal bovine serum to a final concentration of 10% and then incubated for 15 minutes at PH of 7 – 7.6. The content of the decontaminated cell line vial was then be transferred to 9 ml of complete growth medium and spun in a centrifuge at approximately 125 x g for 5 to 7 minutes. Cell pellets were resuspended in a 75 cm<sup>2</sup> flask with the complete growth medium at a culture dilution ratio provided by ATCC for the specific batch of cells we received. Finally, the culture was incubated at 37°C at 5% CO<sub>2</sub> and air 95%. If long term storage is needed at this point, it will require the addition of 5% (v/v) DMSO with the complete growth medium which acts as a freezing medium for the cells and then placed in liquid nitrogen.

To sub-culture the cells, and to remove all traces of serum that contains trypsin inhibitor, the culture medium was discarded, and the cell layer was briefly rinsed with 0.25% (w/v) Trypsin 0.53 mM EDTA solution. We proceeded to add 3.0 ml of Trypsin EDTA solution to the flask and observe the cells under an inverted microscope, usually within 5 to 15 minutes until the cell layer was dispersed. Afterwards, the cells were aspirated gently, and 8.0 ml of complete growth medium was added. Finally, we added the aliquots of the cell suspension to new culture vessels and incubate them at 37°C at 5% CO<sub>2</sub> and air, 95%. If needed, the medium was renewed every 2 to 3 weeks as recommended by the ATCC.

### Milk Digestion

Before we test the effect of the four different kinds of milk that we have chosen, on the proliferation of the triple-negative breast cancer cells, that we have cultured, we must digest them separately via in vitro digestion. This method was taken from two similar studies.<sup>22,23</sup> We took a 10 mL aliquot of commercial cow's, almond and soy milk. The pH of each test solution was adjusted to 2.8 with 6 N HCl, followed by the addition of 0.5

mL of a pepsin suspension (4 g pepsin/100 mL of 0.1 N HCl). The test solutions were incubated and shaking at 37°C for 2 hours. Afterwards, pH of the test solution was adjusted to 5.7 using 5 M NaOH, then 2.5 mL of pancreatin-bile salt mixture (composed of 0.2 g pancreatin and 1.2 g bile salts suspended 100 mL of 0.1 M NaHCO<sub>3</sub>) was added to the test solutions and incubated further for 2 hours. Each test tube will be centrifuged at 4500 RPMs for 10 minutes, room temperature. Following this, we discard the supernatant into 15mL conical tubes to be stored at 4°C, with an aliquot of each test solution passed through a 0.2-µm syringe filter for sterilization. Lastly, we distributed 25, 50, 100, 150 and 250 µL/mL of each of the digested kinds of milk to the sub-cultured wells, along with a phosphate-buffered saline equivalent to each of the five values above, to be incubated at 24 and 48 hours, and counted after each incubation period.

The digested milk was added in different amounts to the cultured cancer cells and incubated for 24 and 48 hours, cell count was noted after incubation, with and without the addition of milk using Alamar blue a cell viability reagent that's not toxic and will detect the metabolic active cells therefore used as a tool to quantitate analysis the cells viability and proliferation.

### Cell Counting and Viability

We followed the instructions provided by the ATCC for this step.<sup>24</sup> A hemocytometer was used for both cell counting and viability. Both was performed after the incubation periods, with and without the addition of milk. Viability requires a mixture of 0.1% erythrosine B dye with PBS in a 1:1 ratio, added to the cell culture and loaded to a hemocytometer. An inverted microscope was used for both cell counting and viability.

### MMT Assays

To showcase that IGF-1 levels in the cancerous cells have increased following the addition of cow's milk, indicating that the cells proliferated due to the presence of IGF-1 in cow's milk, and to see if soy and almond milk had any sort of similar effect, we used a biochemical assay called Human IGF-1 ELISA (enzyme-linked immunosorbent assay) to measure IGF-1 concentration, in the triple-negative breast cancer cells that we have cultured, and compare it to control cells. The IGF-1 was quantified using a

commercial ELISA kit (Abcam, Cambridge, MA, USA).<sup>25</sup>

The total IGF-1 receptors were also measured ELISA kit (Abcam, Cambridge, MA).<sup>26</sup> To sum it up, both assays are quite similar in their procedures; both employ an antibody specifically for either the IGF-1 receptor or human IGF-1, coated on a 96 well plate. To start, all the reagents, samples, and standards was prepared as instructed by the manuals, then the standards, samples, and controls was pipetted into their appropriate wells, as the IGF-1 or IGF-1 receptor from the sample will bound to the wells by the immobilized antibody. Following that, wells were washed, and a biotinylated anti-IGF-1 or IGF-1 Receptor antibody was added, then incubated at room temperature. The unbound biotinylated antibody was washed, afterwards, HRP-conjugated Streptavidin was pipetted into the wells at room temperature and incubated. TMB substrate solution was added after washing the wells. Next, the wells were incubated at room temperature. The color will develop depending on the amount of IGF-1 or IGF1 receptor bound to the wells. Finally, a stop solution was added, it will change colors from blue to yellow and the concentration of the color is measured at 450 nm using a microplate reader.

## RESULTS

### MMT assay to show cell viability

The MMT assay was used to measure cellular metabolic activity which is a sign of cell viability, proliferation and cytotoxicity. The result revealed that viability of the tumor cells sharply increased for milk 1 (A) and milk 2 (B) from the control to 25 uL/mL, which further increased at 50 uL/mL, however, from 100 uL/mL to 200 uL/mL a slight decrease in viability was noticed. This could be due to the media nourishments. On the other hand, the almond and soy milk, not much change was noticed from the control to 20 uL/mL. Soya milk, cell viability continuously decreased with increased concentrations, and almond milk showed the same trend after 50 uL/mL.

### IGF-1 and IGF-1 receptor concentration

Elisa showed an increased number of IGF-1 and IGF-1 receptor concentration in cow's milk 1 (A) groups with the increase in concentration of cow's milk. Comparison of IGF-1 receptors in the

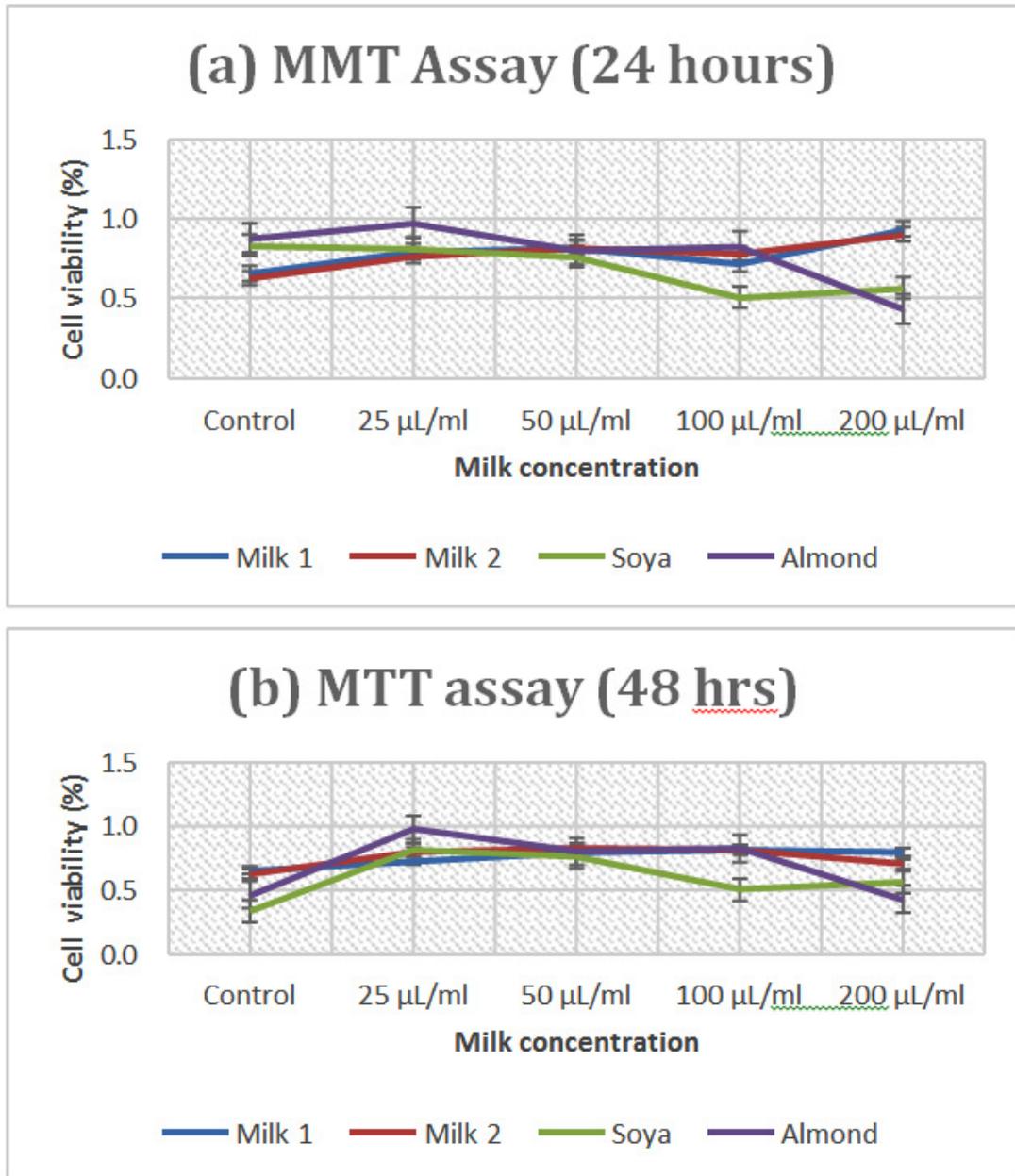
four groups showed gradual increase in milk 1 and Milk 2 groups, Soya milk did not show an increase, however, while almond milk groups C did not show increased levels at higher concentrations. For the Soya milk, there is a peak at 25 uL/mL and 100 uL/mL then sharp decrease at 200 uL/mL. This seems to be an error in the experiment.

## DISCUSSION

Cow's milk is a complex product that has been consumed by humans for decades across all age groups. Doctors often recommend drinking milk during pregnancy, old age especially for women, young school-going children, and during sports activities. Recent studies reported Insulin-Like Growth Factor 1 (IGF-1) in bovine milk as one of the most important components. IGF-1 is not destroyed by pasteurization, and its concentration in milk ranges from 1.0 to 83 ng/mL.<sup>27-28</sup> Treatment with bovine somatotropin (bST) to increase milk production also increases milk concentration of IGF-1<sup>28</sup>. During puberty, mammary gland development relies on IGF-1 for ductal proliferation, with growth hormone-stimulated IGF-1 playing a key role in ductal morphogenesis.<sup>29, 30</sup>

This study agrees with the hypothesis that IGF-1 present in cow's milk is not destroyed by pasteurization and that its use may increase the chances of increasing triple-negative breast cancer (TNBC). In vitro findings revealed that IGF-1 in cow's milk stimulated the proliferation of TNBC cells. The TNBC cell line in culture showed increased growth and proliferation after the addition of cow's milk protein, with growth being proportional to the concentration of the milk. The increase in cell proliferation increases in IGF-1 receptors. In contrast, the addition of almond and soy milk to the culture plates resulted in less prominent and non-proportional increases in cell proliferation, even as the dose increased.

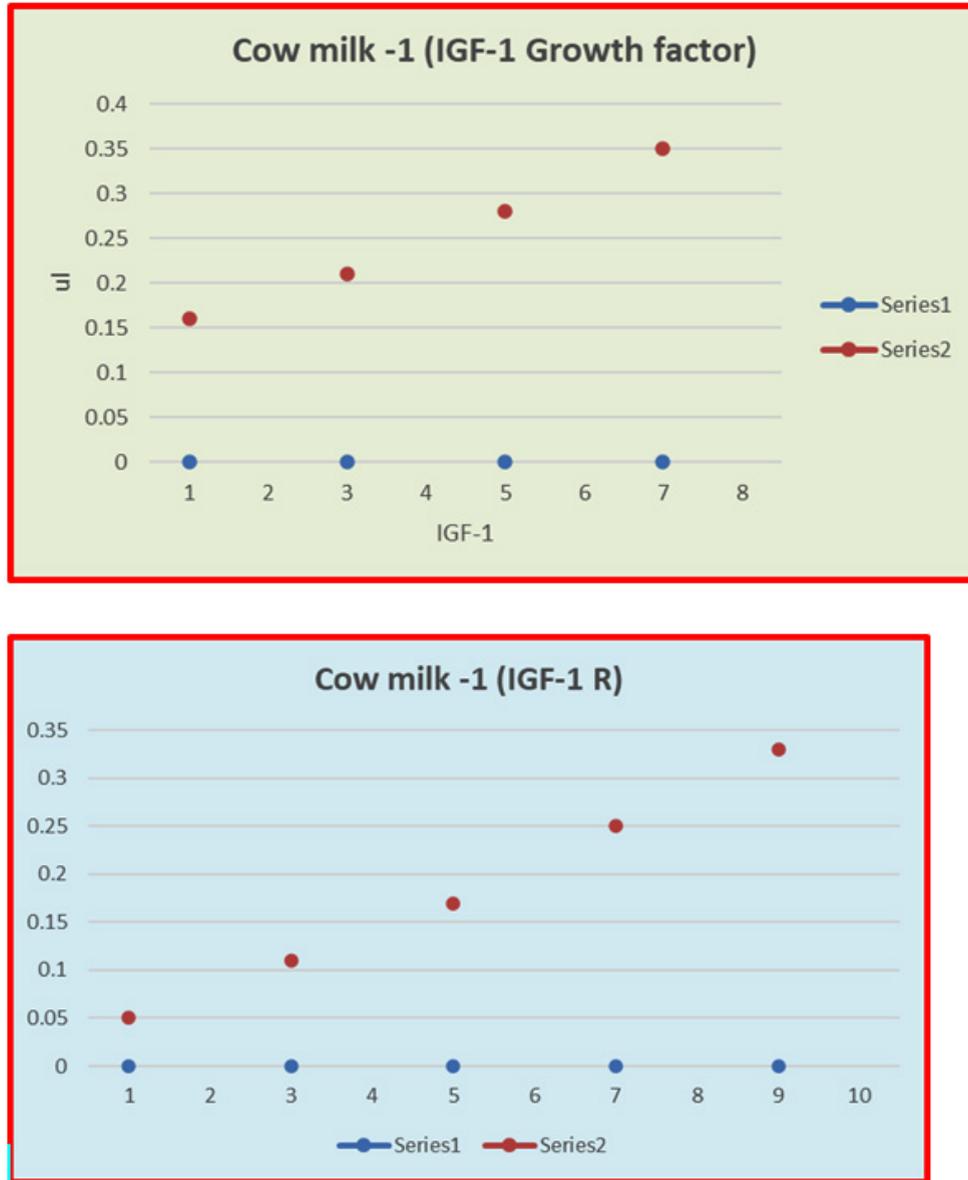
Consumption of cow's milk increases circulating IGF-1 levels in both children and adults.<sup>9, 31-33</sup> Research on 526 individuals revealed that each 200g of cow's milk per day resulted in a 10.0 µg/L increase in IGF-1.<sup>34</sup> Another study from New Zealand reported increased growth in 2-year-old children and increased levels of IGF-1 among those who consumed cow's milk.<sup>35</sup>



**Fig. 1.** a) 24 hours: MMT results revealed that tumor cell viability and metabolic activity increased as the milk concentration was added to the culture plates and was maximum at 200µl/ml; however, for the plant-based milk decline was observed at the highest concentration. b) 48 hours: MMT results revealed similar patterns as seen at 24 hours; tumor cell viability and metabolic activity increased as the milk concentration was added to the culture plates however, for the plant-based milk it increased initially which decline from 100µl/ml and was minimum at the highest concentration

A cross-sectional survey in China reported an increase in breast cancer incidence.<sup>36-37</sup> Kakkoura (2022) also reported a significant positive association between dairy consumption and breast cancer in women. Similarly, a population-based case-control study in Iran confirmed the association between breast cancer and milk intake.<sup>38</sup> Studies

from western Mexico and Uruguay also reported a cow's milk intake and increase incidence of breast cancer.<sup>39-40</sup> Insulin-like growth factor and insulin-like growth 1 receptors (IGF1-R) have been detected in breast tumors and anti-IGF1 treatment was suggested.<sup>41</sup>



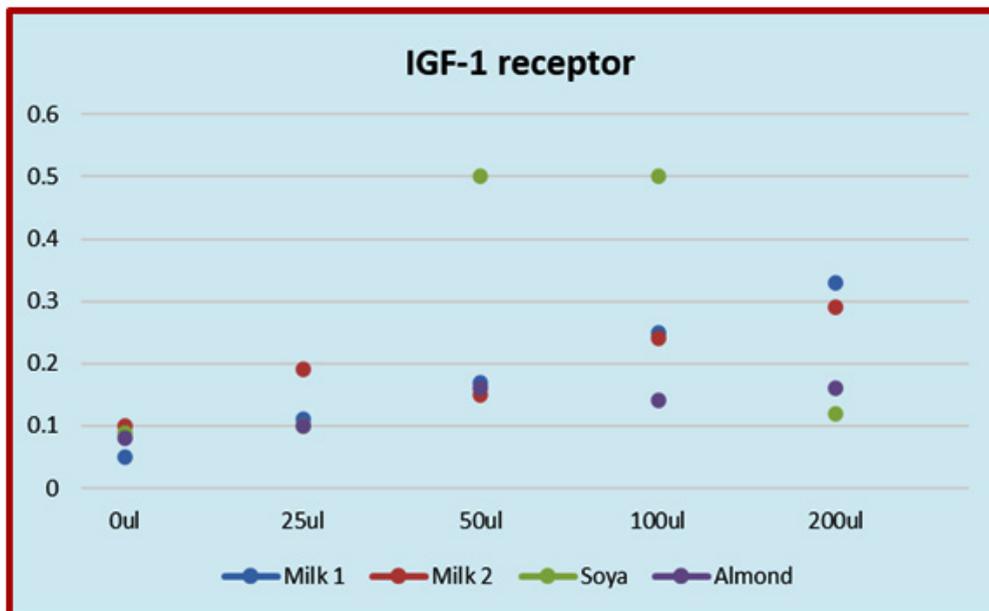
**Fig. 2.** a) The IGF-1 level increased (red dots) proportionately with the addition of cow's milk 1 however, there is no increase in the control (blue dots) b) The IGF-1 receptor level also increased (red dots) proportionately with the addition of cow's milk 1 however, there is no increase in the control (blue dots). Cow's milk 2 had similar results

This study found increased IGF-1 and IGF-1 receptor levels at TNBC cell line MDA-MB-231 after the addition of cow's milk, supporting the theory that cow's milk contains IGF-1, which can affect TNBC cells. MTT assay results revealed increased proliferation of tumor cells in cow's milk groups A and B in vitro.

Another study reported that the IGF signaling pathway is activated in TNBC cell lines, increasing cell proliferation and improving cell survival.<sup>42</sup> It was also reported that estrogen-unresponsive cells show a proliferative response to IGF<sup>43</sup>, although a few earlier studies disagree.<sup>44</sup> IGF receptors were detected in both estrogen receptor-positive (ER+) and triple-negative (TN) cell lines, with higher expression found in TN cell lines.<sup>42</sup> IGF-1 stimulated the proliferation of MDA-MB-231 cell lines, leading to an eightfold increase after 9 days: decreasing the IGF-1 receptor significantly reduced cell growth.<sup>42</sup> It has been reported that IGF-1 ingested through cow's milk elevates circulating IGF-1 levels, which can influence the initiation and proliferation of TNBC cells. Another study reported the role of circulating IGF-1 in breast cancer.<sup>45</sup> A prospective cohort study

in California of 52,795 American women initially free of cancer found that higher consumption of cow's milk was correlated with a ratio of 1.50 for breast cancer over 7.9 years.<sup>46</sup> Another population-based study in Sweden with 16.6 years of follow-up of showed that prolonged milk consumption increased the incidence of breast cancer.<sup>47</sup> Research has shown that drinking cow's milk regularly may cause a measurable increase in circulating IGF-1. For example, a study in Germany found that consuming 200g of cow's milk per day resulted in significant higher levels of IGF-1 in both men and women.<sup>34</sup> A meta-analysis examines multiple studies and concludes that increased serum IGF-1 levels are correlated with a higher incidence of prostate cancer.<sup>48</sup> Some studies have suggested a connection between elevated IGF-1 levels with a higher risk of colorectal cancer.<sup>49</sup> In 2009, FDA concluded that significant levels of IGF-1 from milk would not be able to be absorbed in human hence cow's milk is of no health risk to consumers.<sup>50</sup> However, recent studies have proved otherwise.

Hence elevation of plasma IGF-1 level by intake of cow's milk may increase the circulating GH/IGF-1 during puberty which may increase



**Fig. 3.** The IGF-1 receptor levels increased in the cow's milk 1 and 2 which identifies increase in the tumor cells; plant milk did not show such an increase in the IGF-1 receptor. The soya milk showed higher levels at 50µl/ml and 100µl/ml levels which seems to be an experimental error

the risk of BCa. Elevated IGF-1 levels relate to the development of breast cancer, particularly hormone-independent types like Triple-Negative Breast Cancer (TNBC). The mechanism involves IGF-1 stimulating cell proliferation, inhibiting apoptosis (programmed cell death), and potentially aiding tumor growth and metastasis.

One of the limitations of this study is that the tumor cells were grown in culture, making their growth dependent on the artificial nutritional environment. Nutrient depletion in the culture media can adversely affect tumor cell proliferation and viability. Moreover, tumor cells maintained *in vitro* are prone to genetic and phenotypic drift over time, potentially accumulating mutations or losing key characteristics. As a result, they may adapt to the artificial conditions and no longer accurately represent the original tumor *in vivo*. Additionally, the use of fetal bovine serum (FBS), which is compositionally variable and not physiologically representative of the human microenvironment, introduces experimental inconsistency and limits reproducibility across studies. Commercial milk from two different companies were used in this experiment which may vary in milk collection, quality testing, fortification, pasteurization etc.

### CONCLUSION

This experimental *in vitro* research agrees with the concept that IGF-1 present in cow's milk affects the TNBC cells proliferation and growth *in vitro*, when compared with soy and almond milk. The presence of IGF-1 in cow's milk and its ability to raise circulating IGF-1 levels in humans has sparked considerable interest in its potential role in cancer development, particularly breast cancer. While cow's milk provides essential nutrients and supports growth, its impact on IGF-1 levels and the associated cancer risks are important considerations. IGF-1, on the other hand, is crucial for the growth of the body in children. Plant-based milk alternatives may offer a safer option for those concerned about these risks, although they do not provide the same nutritional profile as cow's milk. Further research is needed to fully understand the connection between dietary IGF-1 intake, circulating IGF-1 levels, and long-term health outcomes, particularly in relation to cancer risk.

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#### Conflict of Interest

The author(s) do not have any conflict of interest.

#### Data Availability Statement

This statement does not apply to this article.

#### Ethics Statement

Approved by the Research and Ethical Committee of the College of Medicine and Health Sciences, National University of Science and Technology (NU/COMHS/EBC0016/2022. This research did not involve human participants, animal subjects.

#### Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

#### Clinical Trial Registration

This research does not involve any clinical trials.

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Not Applicable.

#### Authors' contributions

Mouge Mohammad Salah Al Fragi: Writing of the proposal, performing the experiment and helping the laboratory technician in culturing the cells and other lab procedures; Osama Mohammed Salih Adnan Al Alawi: Assisted in writing the proposal, doing the experiments and writing the manuscript; Mr. Shadhan Alsiyabi: Laboratory in charge of culturing the cells, and the main person responsible of the laboratory procedures including the ELIZA and all the other

experiment techniques; Najam Siddiqi: The main supervisor who received the TRC grant, finalized the proposal, supervised the experimental techniques, procured the ELIZA kits and finally analyzed the results and written the manuscript.

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