Metformin diminishes the unfavourable impact of Nrf2 in breast cancer patients with type 2 diabetes

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Abstract
Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a major regulator of the oxidative stress response and it is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1). The Keap1–Nrf2 axis has a fundamental role in carcinogenesis. In previous studies, the widely used diabetes drug metformin has appeared to have a critical role in the regulation of Nrf2 function. In this study, we assessed the expression of Nrf2 and Keap1 immunohistochemically in 157 patients with type 2 diabetes who underwent breast cancer surgery with curative intent. In total, 78 (49.7%) of these patients were taking metformin alone or combined with other oral anti-diabetic medication at the time of breast cancer diagnosis. We found that high-level cytoplasmic Nrf2 expression predicted dismal overall survival and breast cancer–specific survival, but only in the patients who were not taking metformin at the time of diagnosis. Similarly, low-level nuclear Keap1 expression had an adverse prognostic value in terms of overall survival and breast cancer–specific survival in patients without metformin. On the other hand, high-level nuclear Keap1 expression was associated with prolonged overall survival and breast cancer–specific survival. The results may be explained in terms of non-functioning or displaced Keap1, although more mechanistic pre-clinical and prospective clinical studies are warranted.

Keywords
Oxidative stress, Keap1, Nrf2, metformin, breast cancer

Introduction
Breast cancer is the most common cancer in women and although mortality has decreased as a result of early diagnosis and effective systemic treatments, it still remains the second-most common cause of cancer death in developed countries.¹ Women with type 2 diabetes mellitus (T2DM) have an elevated risk of breast cancer.² In addition, the diagnosis of breast cancer is more likely to be delayed in women with T2DM, and these women have a higher rate of overall mortality than women without the condition.³

Metformin is the standard first-line therapy for T2DM.⁴ It has anti-mitotic, anti-angiogenic and anti-inflammatory properties.⁵ It decreases lipogenesis and reduces blood glucose concentrations by increasing the insulin sensitivity of tissues and lowering levels of insulin-like growth factor-1 (IGF1). In combination with chemotherapy, metformin can also affect survival by enhancing cytotoxicity and by increasing radiosensitivity.

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in breast cancer cells. Metformin also decreases breast cancer cell survival by increasing levels of reactive oxygen species (ROS), which leads to DNA damage and apoptosis. There have been several epidemiological studies on metformin use in association with breast cancer risk and survival, but evidence of a clear correlation is still inconclusive.

The production of ROS is an inevitable consequence of aerobic metabolism. Without appropriate antioxidant defence, oxidative stress can occur, and this can lead to oxidative stress–related diseases including diabetes and cancer. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a constantly synthesized Cap’n’Collar transcription factor and a major regulator of the oxidative stress response. During unstressed conditions, Nrf2 is found at low levels, since in cytoplasm it is constantly subjected to proteosomal degradation via a Kelch-like ECH-associated protein 1 (Keap1)-dependent mechanism. Keap1 works as an adaptor subunit of Cullin 3-based ubiquitin E3 ligase. However, under oxidative stress, Keap1 is detached from Nrf2, which then transfers to the nucleus and binds to antioxidant response elements (AREs) for activation. Under oxidative stress, several Cys residues in Keap1 are oxidized, Nrf2 becomes stable and avoids degradation, which ultimately results in widespread expression of antioxidant proteins. Nrf2 favours survival and proliferation of tumour cells by inducing chronic activation of an ARE-mediated cytoprotective reaction that leads to tumour-cell adaptation to an oxidative environment. It can prevent carcinogenesis by inducing the destruction of ROS arising from chemical action or ultraviolet radiation. If properly regulated, Nrf2 protects against cancer and inflammation. However, Nrf2 acts as both a tumour suppressor and a tumour promoter. It can accelerate carcinogenesis, metastasis and resistance to radiotherapy and chemotherapy. This non-beneficial activation of Nrf2 is caused by somatic mutations, epigenetic factors and oncogenic signalling alterations.

Mounting evidence suggests that the Keap1–Nrf2 axis has a fundamental role in carcinogenesis, and metformin has been depicted as a critical regulator of Nrf2 function. Therefore, we investigated the role of metformin treatment in overall survival (OS) and breast cancer–specific survival (BCSS) and their association with Keap1 and Nrf2 protein expression in a cohort of T2DM patients suffering from breast cancer.

### Materials and methods

#### Study population

Our study population consisted of women with type 2 diabetes who underwent operation for breast cancer with curative intent at Oulu University Hospital, Finland, in 2007–2016. Data on patients were obtained from Oulu University Hospital records. These hospital records included T2DM diagnostic data, information on anti-diabetic medication (ADM), and the patient’s age and body mass index (BMI). Information obtained from hospital records on breast cancer included stage, histological data, receptor status (oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2)), the proliferation marker Ki-67, recurrence time, BCSS and OS. Using personal identity codes (PICs), we were able to link these records to each patient.

Classification of different ADM groups was based on information on ADM used at the date of breast cancer operation. The patient was classified as a metformin user if she had used metformin alone or in combination with some other oral ADM. Patients were classified as non-metformin users if they used only other forms of oral ADM or insulin (including insulin combined with metformin), or were non-ADM users.

Of 157 patients (and 179 breast cancer tumours), there were 78 in the metformin group, of whom 57 used only metformin and 21 used metformin combined with another type of oral ADM. The non-metformin user group consisted of 15 women who used only insulin, three who used another type of oral ADM only, 17 women who used insulin combined with metformin, 10 women who used insulin combined with another type of oral ADM and 10 women who used insulin combined with both metformin and another type of oral ADM. A total of 24 patients did not use any ADM (Figure 1).

Follow-up of the study population began at the date of breast cancer operation, except for patients who were first treated with neoadjuvant chemotherapy (n = 4). In those cases, the start of follow-up was the date of diagnosis of breast cancer. Follow-up ended at the time of death or closure of the follow-up period (22 March 2017). The median follow-up time was 37.0 months.

#### Immunohistochemistry

Breast cancer tissue sections of 3–4 μm (for Keap1) and 4.5 μm (for Nrf2) were cut from representative paraffin blocks and placed on SuperFrostPlus glass slides (Menzel-Gläser, Germany). They were de-paraffinized in xylene and rehydrated in a descending series of ethanol solutions, incubated in citrate buffer at pH 6.0, then heated in a microwave oven for 12 min and cooled at
room temperature before adding the primary antibody. Rabbit polyclonal antibodies (Abcam, Cambridge, UK) for Keap1 (ab66620) and Nrf2 (ab76026) were used at dilutions of 1:800 and 1:600, respectively. A NovoLink Polymer Detection System (Leica Biosystems Newcastle Ltd) was used for detection of Keap1 and Use Envision kits (Dako, code K500) for detection of Nrf2. For negative controls, the primary antibodies were replaced with phosphate-buffered saline and serum isotype controls. Immunoreactivity was assessed by three observers (J.K., P.K., U.P.). Both nuclear Keap1 (Figure 2) and cytoplasmic Nrf2 (Figure 3) were divided into two categories, present and absent, according to immunoreactivity. Nuclear Nrf2 was categorized as 0%–100%, rounding to the nearest 10%. In statistical analyses, a cut-off point of 30% (median) was applied, immunoreactivity under 30% being categorized as negative and ≥30% being categorized as positive (Figure 3). Cytoplasmic Keap1 was divided into four categories (0–3) according to immunoreactivity, but for further statistical analysis, expression levels were classified as 2-class variables (0–1 as negative and 2–3 as positive) (Figure 2). Expression of Ki-67 was divided into 0%–15% or >15%, and grade was either grade I–II or grade III in the statistical analyses.

Statistical analysis
Statistical analysis was performed using IBM SPSS Statistics software, v. 23.0.0.0 (IBM Corporation, Armonk, NY, USA). T-class was divided in statistical analyses to either T1 or T2–4 and nodal status to either positive or negative. The significance of associations was assessed using two-sided Pearson’s chi-square tests. Kaplan–Meier curves with the log-rank test were applied in survival analysis. Survival times were calculated from the time of diagnosis to the time of confirmed breast cancer–related death (BCSS) or time of death resulting from any cause (OS). Patients with multifocal tumours were excluded from survival analyses, since Keap1 and Nrf2 expression levels showed variation in different tumour deposits. Cox regression analysis was applied in multivariate analysis, where the most important traditional prognostic factors, N-class (N0 or N1–3) and T-class (T1 or T2–4) were included in the model. In all statistical analyses, p-values less than 0.05 were considered significant.

Results
The mean age of the metformin users was 68.4 years (range, 45–89 years) and that of non-metformin users 71.3 years (range, 40–94 years). Neoadjuvant therapy was used in only two patients in both groups. Stage and presence of multifocal disease did not differ in metformin users and non-users. Patients in the metformin group had a lower BMI than those in the non-metformin group, 30.4 and 33.0 kg/m², respectively (Mann–Whitney U test, p = 0.029; Table 1).

Tumour histology, receptor status and Ki-67 expression levels were similar in both groups. Cytoplasmic Keap1 expression was observed in 46.1% of the tumours in the metformin group and in 34.4% of the tumours in non-metformin group. Nuclear Keap1 was present in 33.7% of the tumours in the metformin group and in 30.0% of the tumours in the non-metformin group. Cytoplasmic Nrf2 was positive in only 13.5% of the tumours in the metformin group and in 30.0% of the tumours in the non-metformin group. Nuclear Nrf2 was present in 33.7% of the tumours in the metformin group and in 30.0% of the tumours in the non-metformin group. Cytoplasmic Nrf2 was positive in only 13.5% of the tumours in the metformin group and in 12.2% of the tumours in the non-metformin group. Nuclear Nrf2 was positive in 50.6% of the tumours in the metformin group and 41.1% of the tumours in the non-metformin group (Table 2).
Figure 2. Immunohistochemical staining of Keap1 in breast cancer tissue, 100-fold magnification. (a) Cytoplasmic Keap1 graded as 1 (negative) and nuclear Keap1 absent. (b) Cytoplasmic Keap1 graded as 2 (positive) and nuclear Keap1 present. (c) Cytoplasmic Keap1 graded as 3 (positive) and nuclear Keap1 absent.

Figure 3. Immunohistochemical staining of Nrf2 in breast cancer tissue, 100-fold magnification. (a) Both nuclear and cytoplasmic Nrf2 are absent. (b) Nuclear Nrf2 is present and cytoplasmic Nrf2 is absent. (c) Both nuclear and cytoplasmic Nrf2 are present.
Association of Nrf2 and Keap1 with traditional prognostic factors

Positive nuclear Keap1 expression was associated with a more benign prognostic factor profile, including ER-positive tumours \((p = 0.035)\), HER2 negativity \((p = 0.003)\), better differentiation \((p = 0.0001)\) and lower proliferation rate \((p = 0.00001)\). Accordingly, cytoplasmic Keap1 was connected with PR positivity \((p = 0.027)\), low grade \((p = 0.0014)\) and low-level proliferation \((p = 0.0071)\). Nuclear Nrf2 was associated with the presence of multifocal disease \((p = 0.025)\). Nuclear Nrf2 was not associated with Keap1 expression, but cytoplasmic Nrf2 was associated with lower-level cytoplasmic and nuclear Keap1 expression \((p = 0.042\) and \(p = 0.032\), respectively). Metformin treatment was not associated with traditional clinicopathological factors of breast cancer.

Association of Nrf2 and Keap1 with survival, in relation to metformin use

Stronger cytoplasmic Nrf2 expression predicted dismal OS \((p = 0.038)\) in univariate analysis in patients without metformin treatment but not in those in the metformin group \((p = 0.48, \text{Figure 4(a) and (b)})\). This was also observed in terms of BCSS \((p = 0.029\) and \(p = 0.48\), respectively; \text{Figure 4(c) and (d)})

| Table 1. Patient characteristics in metformin users and non-metformin users. | Table 2. Tumour characteristics in metformin users and non-metformin users. |
|---|---|
| Age | Metformin users \((n = 78)\) | Non-metformin users \((n = 79)\) |
| Mean (years) | 68.4 | 71.3 |
| Min-max | 45–89 | 40–94 |
| BMI | Metformin users \((n = 78)\) | Non-metformin users \((n = 79)\) |
| Mean | 30.4 | 33.0 |
| Min-max | 20.4–46.7 | 17.2–65.6 |
| Stage | Metformin users \((n = 78)\) | Non-metformin users \((n = 79)\) |
| Local | 42 (53.8%) | 50 (63.3%) |
| Advanced | 36 (46.2%) | 29 (36.7%) |
| Multifocal disease | Metformin users \((n = 78)\) | Non-metformin users \((n = 79)\) |
| Yes | 10 (12.8%) | 9 (11.4%) |
| No | 68 (87.2%) | 70 (88.6%) |
| Survival status of the patient | Metformin users \((n = 78)\) | Non-metformin users \((n = 79)\) |
| Alive without recurrence | 57 (73.1%) | 61 (77.2%) |
| Alive with recurrence | 6 (7.7%) | 1 (1.3%) |
| Death from breast cancer | 8 (10.3%) | 5 (6.3%) |
| Death from other cause | 7 (9.0%) | 11 (13.9%) |
| Unknown | 0 (0%) | 1 (1.3%) |

BMI: body mass index.

**Association of Nrf2 and Keap1 with traditional prognostic factors**

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**Association of Nrf2 and Keap1 with survival, in relation to metformin use**

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In Cox regression analysis, cytoplasmic Nrf2 was a significant prognostic indicator of OS (relative risk \((RR)\), 4.246; 95% confidence interval \((CI)\), 1.015–17.756; \(p = 0.048\)) in the non-metformin patient group when nodal status \((RR, 3.133; 95\% CI, 0.744–13.189; p = 0.119)\) and tumour size \((RR, 6.062; 95\% CI, 1.182–31.091; p = 0.031)\) were included in the model. Intriguingly, cytoplasmic Nrf2 expression \((RR, 4.940; 95\% CI, 1.144–21.337; p = 0.032)\) was also the most important prognostic factor in terms of BCSS (nodal status: \(RR, 4.560; 95\% CI, 0.882–23.583; p = 0.070\); tumour size: \(RR, 5.763; 95\% CI, 1.094–30.358; p = 0.039, \text{Table 3})\). Low-level nuclear Keap1 expression...
was associated with unfavourable outcome in terms of OS ($p = 0.028$) and BCSS ($p = 0.041$) in non-metformin users (Figure 5(a) and (c)) but not in metformin users (Figure 5(b) and (d)). However, in multivariate analysis, nuclear Keap1 expression was not statistically significant.

We also performed survival analysis with a four-classed variable where groups were created according
to nuclear Keap1 and cytoplasmic Nrf2 expression in metformin users and non-users (Figure 6). Dismal OS and BCSS times were observed in patients with negative nuclear Keap1 and positive cytoplasmic Nrf2 immunohistochemistry ($p = 0.021$ and 0.028, respectively; analyses comparing all four groups) in the non-metformin group, but not in the metformin group ($p = 0.76$ for both OS and BCSS).

Metformin medication itself had no impact on BCSS in patients with T2DM. OS was (non-significantly) longer in the patients with metformin treatment ($p = 0.058$). In multivariate analysis, this association remained statistically non-significant. BMI was not associated with OS or BCSS in either medication group.

**Discussion**

Stronger cytoplasmic Nrf2 expression predicted dismal OS and BCSS in T2DM patients without metformin treatment but not in patients with metformin treatment. Low-level nuclear Keap1 expression was also associated with unfavourable outcome in terms of OS and BCSS in non-metformin users in our study. Hence, it is plausible that metformin modifies the unfavourable balance of Keap1/Nrf2 which seems to be associated with cancer-specific and overall survival.

Metformin decreases energy status, which results in activation of AMP-activated kinase (AMPK).^{19} This leads to decreased serum concentrations of insulin and IGF-1.^{19} AMPK both directly and indirectly inhibits the signalling of PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)/protein kinase B (AKT)/mamalian target of rapamycin 1 (mTORC1).^{19} mTORC1 inhibition leads to a decrease in global protein synthesis and lipogenesis.^{19} In addition, AMPK activation by metformin may subsequently lead to the activation of Nrf2.^{13,20,21} In addition, various in vitro studies have shown that metformin reduces Nrf2 messenger RNA
(mRNA) and protein levels via a Keap1-independent mechanism by attenuating Raf (proto-oncogene serine/threonine-protein kinase)-ERK (extracellular signal-regulated kinase) signalling in various cancer cells and this suppresses Nrf2 signalling. In endometrial cancer cells, metformin treatment blocks Nrf2 hydroxymethylation, which leads to the elimination of chemoresistance of endometrial cancer cells. In our study, there was no association between nuclear or cytoplasmic Nrf2 expression and metformin treatment. Therapeutically used metformin levels are much lower than those used in in vitro studies and redox-state regulation may be considered to be much more complex in vivo than in vitro, which may explain this contradiction.

Nrf2 expression in relation to patient outcomes has been extensively studied in connection with solid carcinomas. In a recent meta-analysis concerning 17 original studies of various cancers, including one breast cancer study, it was concluded that Nrf2 expression had a worsening impact on OS (hazard ratio, 2.29). In breast cancer patients, nuclear Nrf2 expression has previously been linked to higher grade and rapid proliferation, and in our study, to the presence of multifocal disease. In a previous study, Nrf2 also exceeded the prognostic power of nodal status. Likewise, our results suggest that cytoplasmic Nrf2 expression and nuclear Keap1 expression may be highly important prognostic factors in diabetic breast cancer patients, with prognostic power exceeding that of nodal status. Notably, no difference in survival in relation to the level of Nrf2 expression was observed in patients with metformin treatment. Our results are in line with those in a study carried out by Onodera et al., where high-level

**Figure 6.** Kaplan–Meier curves demonstrate associations between the combination of nuclear Keap1 and cytoplasmic Nrf2 expression, and survival. (a) OS in non-metformin users. (b) OS in metformin users. (c) BCSS in non-metformin users. (d) BCSS in metformin users. Crosses indicate censored cases.
Nrf2 expression was associated with worse disease-free survival. However, in our study, only cytoplasmic Nrf2 predicted worse outcome, which has also been observed in other types of carcinomas. Under unstressed conditions, cytoplasmic Nrf2 is maintained at low levels, with Keap1-mediated degradation. Thus, high-level cytoplasmic Nrf2 expression found in the patients with the worst prognosis may reflect non-functioning Keap1. In addition to survival connections, cytoplasmic Nrf2 expression was associated with markers of high-level disease aggressiveness (ER- and PR-negativity, high grade and proliferation index), while this was not seen with nuclear Nrf2. An association between Nrf2 expression, grade and Ki-67 has been reported in a smaller breast cancer study. In addition, oestrogen has previously been demonstrated to induce Nrf2 synthesis and downstream protein synthesis, at least in ER-positive breast cancer cell lines.

The absence of nuclear Keap1 in our study was an extremely powerful prognostic factor, with prognostic value exceeding that of nodal status. To the best of our knowledge, the possible direct interaction between Keap1 and metformin in breast cancer has not been reported before. However, in one study, metformin has been shown to be protective against myocardial ischaemia via a Keap1-mediated mechanism. Interestingly, in our study, a trend towards better outcome was noted with higher nuclear Keap1 expression together with metformin medication. We have recently reported that nuclear Keap1 expression is associated with longer disease-free survival in patients with locally advanced inoperable breast carcinomas. In addition, studies on pancreatic cancer and squamous non-small-cell lung carcinoma have revealed better outcome in connection with Keap1 protein overexpression. On the other hand, Keap1 expression correlated with worse BCSS in our previous material enriched with triple-negative breast cancers, but only in univariate analysis.

The overall prognosis of the diabetic patients in our study cohort was relatively low, with only 71.6% of patients surviving at 5 years. The observed results in survival analysis would have been even more impressive if multifocal tumours (24% of all tumours) had been included in the survival analyses. To ensure the reliability of the results, however, this was not done. The available medication data were that at the time of surgery only, without certainty that the patients continued metformin treatment during the follow-up period. This, along with the relatively short follow-up period, can be considered as a limitation of the study.

Conclusion

High-level expression of cytoplasmic Nrf2 predicts poorer survival in breast cancer patients with T2DM without metformin treatment. In addition, nuclear Keap1 expression overrides nodal status and tumour size as a prognostic factor in this cohort of breast cancer patients. We hypothesize that metformin may modify the balance of Keap1/Nrf2, which seems to be associated with cancer-specific and overall survival.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

This study was approved by the local ethics committee of Oulu University Hospital (20.4.2015 § 102) and the National Supervisory Authority of Welfare and Health (1237/06.03.01/2015).

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