Unravelling the link between diabetes, insulin treatment and breast cancer

Heleen K. Bronsveld

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Unravelling the link between diabetes, insulin treatment and breast cancer

Ontrafelen van de link tussen diabetes, insuline behandeling en borstkanker

(met een samenvatting in het Nederlands)

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Voor mijn moeder

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Chapter 1

Introduction and outline

Diabetes mellitus

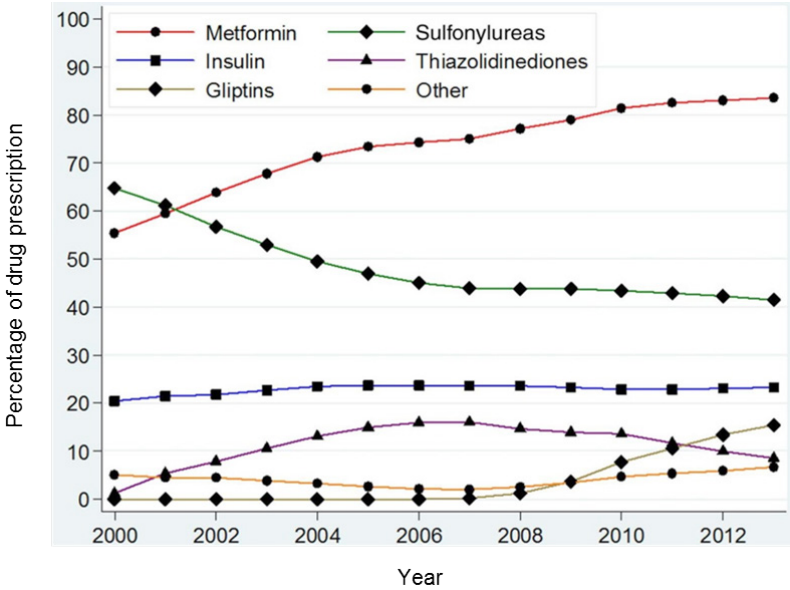
Diabetes mellitus is a major global health problem with increasing prevalence in women. The global age-standardized prevalence of diabetes mellitus (type 1 and type 2), further referred to as diabetes, among women has increased from 5% in 1980 to 8% in 2014 [1]. Changes in lifestyle patterns, such as increased high-caloric diet and decreased physical activity, which result in obesity, fueled this increase [2]. Together with population growth and ageing, this rise in diabetes prevalence has led to a nearly four times higher number of patients living with diabetes worldwide in these 35 years [1].

Diabetes is a chronic metabolic disorder. Diabetes is characterized by chronic hyperglycemia (elevated blood glucose levels) if untreated or undertreated, resulting from failure to produce, secrete, and/or use insulin efficiently [3]. Diabetes is typically divided in two major subtypes. Type 1 diabetes accounts for only 5-10% of all cases, while type 2 diabetes is prevalent in 90-95% of all cases [4]. Type 1 diabetes usually presents during childhood or adolescence and is an auto-immune disease with an acute onset, caused by the destruction of the insulin-producing beta cells in the pancreas. Since the body can no longer produce insulin, patients with type 1 disease depend on lifelong insulin treatment for their survival. Main risk factors for type 1 diabetes are genetic predispositions and environmental factors such as infections and intestinal microbiota [5].

Type 2 diabetes is characterized by insulin resistance and relative impairment of insulin secretion, with a slow onset of the disease and diagnosis in late adulthood. Risk factors for type 2 diabetes are excess body weight, physical inactivity, poor nutrition and older age [4]. In an early stage of the disease, the pancreas increases secretion of insulin to compensate for decreased insulin sensitivity of body tissue resulting in hyperinsulinemia (elevated blood insulin levels) [3]. In this stage, type 2 diabetes is often managed by dietary changes and increased physical activity. If the disease progresses, medication is required to lower blood glucose levels. Metformin is currently the recommended initial glucose-lowering drug. If blood glucose levels remain poorly controlled, other medication is added, such as sulfonylureas, and eventually insulin is prescribed (Figure 1) [6-8]. Although only ~20% of the type 2 diabetes patients, usually the elderly, are treated with insulin, this group of patients is the vast majority of insulin users [7].

Women with diabetes, both type 1 and type 2, are at risk of developing a range of dangerous and costly complications, especially when un(der)treated, such as microvascular and macrovascular diseases of e.g. kidneys and heart. Diabetes has also been associated with increased risk of (breast) cancer [9]. All of these complications can endanger women's health and survival, which makes the burden of the disease high [10].

Figure 1. Prevalence of prescription of different antidiabetic medications among patients with type 2 diabetes treated with antidiabetic drugs (Data source: reference [7])

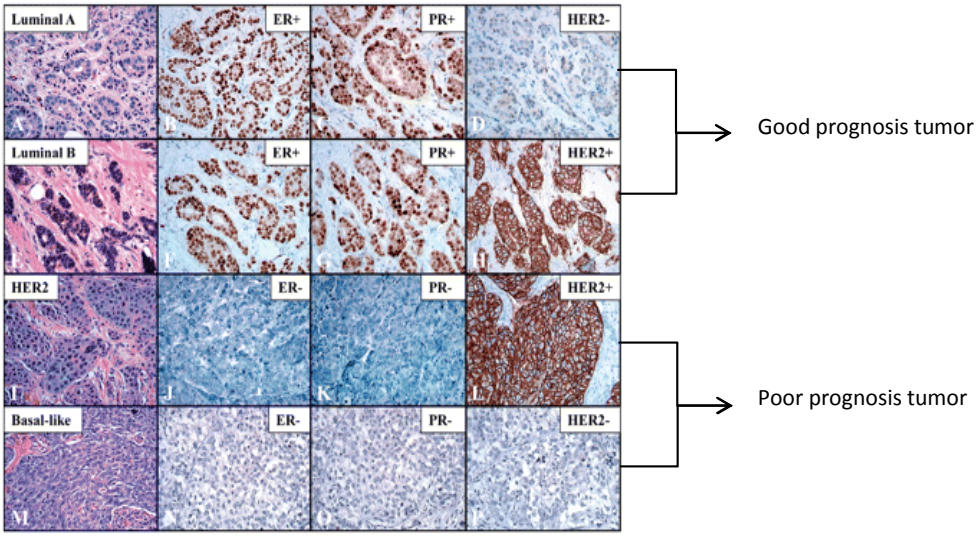


Breast cancer

Breast cancer is the most prevalent cancer in women with 1.67 million new cases diagnosed in 2012 worldwide [11]. The number of breast cancer cases diagnosed each year has increased almost three times since 1980 and lifetime risk for breast cancer is 5.6% among women worldwide [12]. Furthermore, the number of women living with breast cancer is increasing due to ageing of the population and lower mortality rates due to better treatment [11, 13].

Breast cancer is no longer considered as one single disease, but as a disease of different subtypes with possibly a different etiology. Gene expression of breast cancer tumors has resulted in the identification of four molecular subtypes of the disease; luminal A, luminal B, Hormone Epidermal growth factor Receptor 2 (HER2) enriched and triple negative/ basal-like tumors [14]. Those subtypes reflect biological diversity and were shown to be associated with different clinical outcome and prognosis [15]. Classically, sub-classification of breast tumors is accomplished by immunohistochemical staining of tumor tissue for Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal growth factor Receptor 2 (HER2) (Figure 2) and to date, this is still the most frequently used method to guide treatment decisions. Luminal tumors are mostly ER positive; tumor growth largely depends on ER signaling and therefore treatment of these tumors often include hormone therapy such as tamoxifen [16]. Women with luminal tumors have relatively high survival rates compared to the other subtypes. In contrast, triple negative tumors, of which basal-like is a subset, do not express hormone receptors and are often aggressive tumors with a poor prognosis [15]. HER2 enriched tumors are often HER2 positive, and mostly ER and PR negative [16].

Figure 2. Breast cancer clinical subtypes based on differential immunohistochemical staining of ER, PR and HER2 (Data source: adapted from reference [17])



Risk factors for breast cancer are older age, genetic susceptibility due to mutations in e.g. *BCRA1* or *BCRA2* genes or family history of breast cancer, and reproductive factors, such as an early menarche, late or no pregnancy and late menopause [18]. Additionally, lifestyle factors such as physical inactivity, being overweight or obese after menopause, drinking alcohol and smoking also increase risk of breast cancer [18, 19]. Some of these reproductive factors (age at menarche, age at first birth and parity) and lifestyle factors (BMI and alcohol consumption) have also been associated with the development of certain breast cancer subtypes in meta-analyses [20-22]. Biological differences exist between breast cancer arising in premenopausal and postmenopausal women and therefore these associations may vary by menopausal status; e.g. obesity is associated with the development of hormone receptor negative breast cancer in premenopausal women, whereas in postmenopausal women, adiposity was associated with PR-positive tumors [21]. Studies investigating the association between diabetes and breast cancer subtypes are scarce.

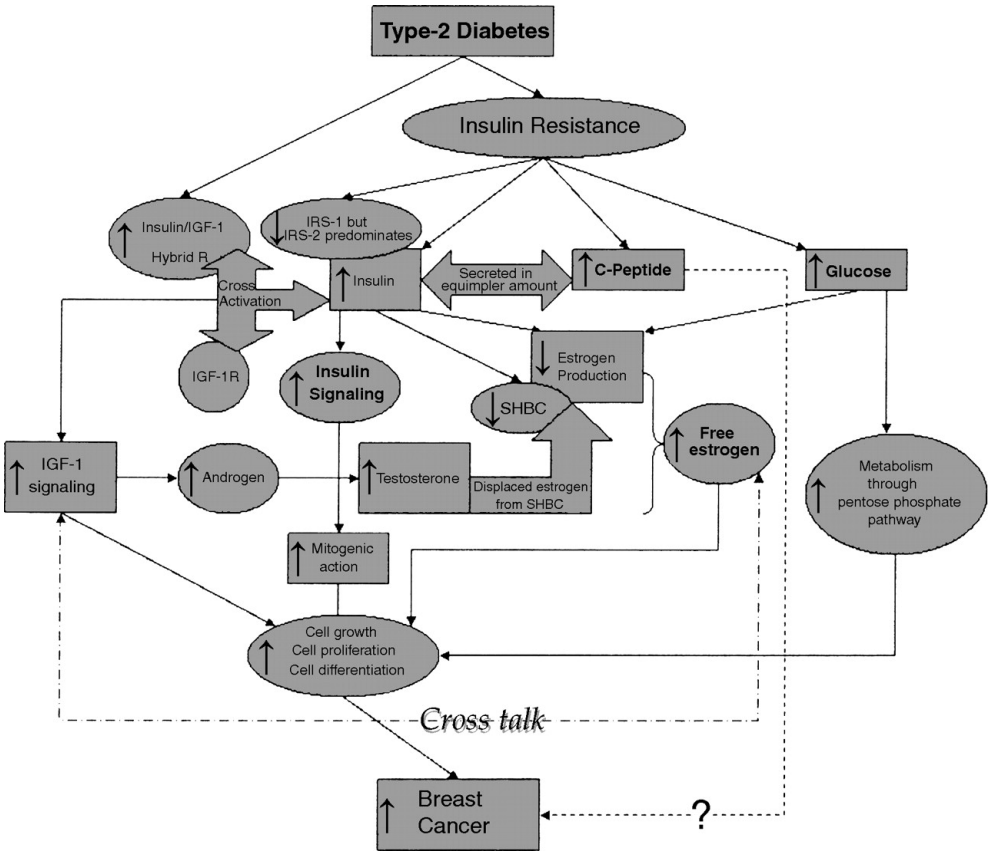
Diabetes and breast cancer

Breast cancer and diabetes are diagnosed within the same individual more frequently than expected by chance, even after adjustment for age. Meta-analyses reported that women with diabetes have a 20% increased risk of developing breast cancer [9, 23]. The exact mechanisms underlying the association between diabetes and breast cancer are unknown. Several mechanism have been proposed in literature (Figure 3) [23, 24].

In women with type 2 diabetes, the disease itself might have a direct effect on tumor growth due to physiological effects of hyperglycemia, or might be a marker of underlying biological factors

that alter breast cancer risk such as insulin resistance and hyperinsulinemia [23-25]. In addition, diabetes treatment has been associated with breast cancer; insulin potentially increases risk of breast cancer [26], while metformin use would potentially decrease risk [27]. The potential mechanisms underlying the associations between diabetes treatment and breast cancer are discussed in the next paragraph. Another explanation for the association between type 2 diabetes and breast cancer is that these diseases share several risk factors including obesity, a sedentary lifestyle, high caloric diet, and ageing, and therefore these women are more likely to develop both diseases [23, 25]. These factors are generally interrelated, which complicates assessing the causal effect attributable to specific risk factors. It might also be that there are shared genetic risk factors.

Figure 3. Potential mechanisms for the influence of type 2 diabetes on breast cancer (Data source: reference [23])



Physiological conditions related to type 2 diabetes may influence cell growth, cell proliferation and cell differentiation via changes in signaling of growth factors (insulin and insulin growth factor), via altered levels of circulating estrogens and androgens and through glucose metabolism via the pentose phosphate pathway creating a microenvironment favorable for tumor development. IGF-I=insulin like growth factor 1,IRS= insulin receptor substrate. IGF-1R=IGF-I receptor, SHBC=sex hormone–binding globulin.

Diabetes, is believed not only to be a moderate risk factor for breast cancer, but it also has been shown that overall mortality after breast cancer diagnosis is 50% higher as compared to women without diabetes [28]. It is unclear whether the higher overall mortality is related to a poorer prognosis specific to breast cancer [9]. Diabetes itself and its complications may also increase the risk of overall mortality [29] and shared cancer-promoting factors in patients with diabetes increases the risk of death from competing causes (metabolic/cardiovascular disease). However, it could be that the poorer survival among women with diabetes is mediated by alterations in tumor tissue and hormone sensitivity, resulting in the development of a more aggressive or less treatment-responsive tumor subtype. At the time of the start of the studies in this thesis, no data existed investigating such potential associations.

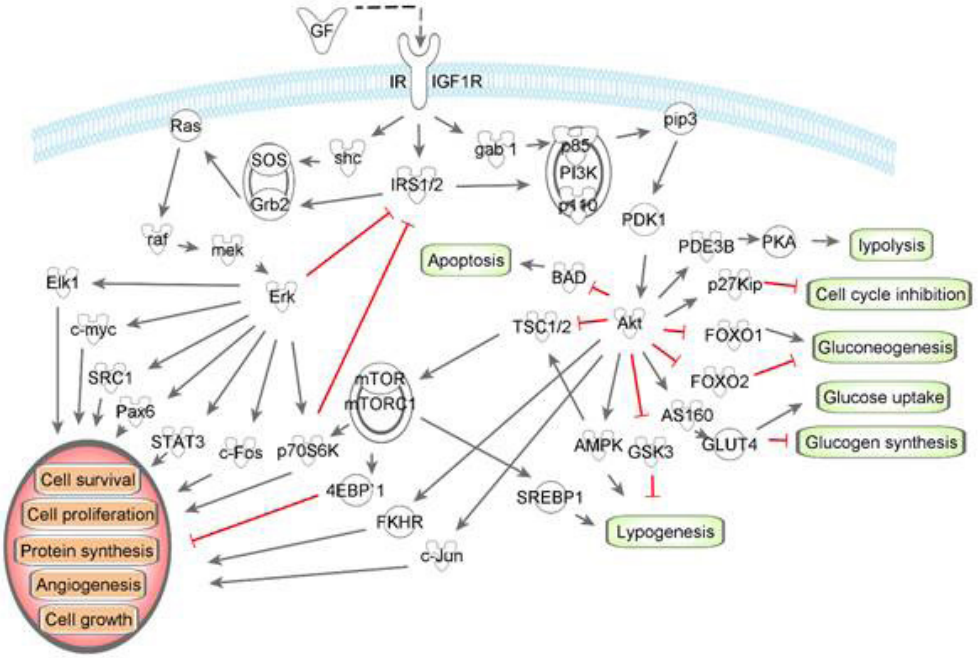
Insulin and breast cancer

Insulin can act as a growth factor, and it is biologically plausible that high levels of endogenous insulin and/or exposure to exogenous insulin or insulin analogues, could stimulate neoplastic growth [24, 30]. Since breast tumors are hormone-driven, it is possible that insulin may be a driver of tumor growth in breast tissue specifically. There is experimental support that insulin interacts with estrogens and might stimulate tumor growth via the ER pathway (Figure 3) [24, 31]. The most plausible hypothesis concerning the mechanism underlying the potential link between insulin and tumor growth is that these act through the insulin (INSR) and insulin like growth factor 1 receptors (IGF1R) (Figure 4), to stimulate cell growth and inhibit apoptosis [32]. It has been shown that INSR and IGF1R are overexpressed in breast cancer tissue [24].

Opposed to insulin, metformin has been shown to possess tumor suppression abilities; including decreased INSR and IGF1R signaling (Figure 4), inhibition of mammalian target of rapamycin (mTOR), and activation of adenosine monophosphate-activated protein kinases (AMPK) [33, 34]. Metformin is a biguanide, and is considered to increase insulin sensitivity and to decrease circulating insulin.

Over the past years, several concerns have been raised regarding the safety aspect of insulin analogues. Insulin analogues are structurally transformed from human insulin, to have an altered pharmacokinetic profile, however, this may result in different binding affinity towards the IGF1R [36]. Differences between mitogenic properties of different insulin analogues have been tested in different mammary cell lines [37]. Although insulin glargine appears to have the most mitogenic properties *in vitro* [36, 38], it is not clear how these results can be extrapolated to breast cancer risk in clinical practice.

Figure 4. The INSR and IGF1R signaling pathway (Data source: reference [35])



Activation of the INSR or IGF1R by a growth factor (GF) such as insulin causes auto-phosphorylation. This activates two intracellular signaling pathways: mitogen-activated protein kinase (MAPK-ERK) and phosphatidylinositol 3-kinase (PI3K-AKT). Activation of the IGF1R predominantly stimulates the MAPK-ERK pathways, while the INSR mainly activates the PI3K-AKT pathway. The PI3K plays a role in glucose metabolism, whereas MAPK lead to effects associated with mitogenesis. However, as shown, there are many cross links between the MAPK-ERK and PI3K-AKT pathways making the INSR/INSR signaling pathway complex.

Several studies have linked the use of insulins to the occurrence of cancer. However, many of these studies suffered from methodological limitations, and results have been conflicting. In 2009, four epidemiological studies raised concern that insulin analogues, especially insulin glargine, might increase risk of cancer [39-42]. Although the results were inconsistent and the authors stressed the limitations of their studies, this lead to an urgent call by the European Medicines Agency (EMA), responsible for the safety assurance of medicine in Europe, for further in-depth evaluation [43, 44]. In 2011, the "CAncer Risk and INsulin analoGues" (CARING) project [45], funded by the Seventh Framework Program of the European Commission, was initiated. The overall objective was to quantify the risk of cancer associated with the use of insulin and insulin analogues using a multi-country database with a proper design, large patient populations, and a long follow-up. In addition, the CARING project aimed to further address biological mechanisms of cancer risk associated with diabetes and insulin use. Acknowledging that such biological mechanisms may be different for different cancers, this thesis focusses on breast cancer development specifically.

Thesis objectives

The overall aim of this thesis is to unravel the link between diabetes, insulin (analogues) and breast cancer risk and breast cancer subtypes. More specifically, this thesis concentrates on potential mechanisms of breast cancer initiation and/or promotion in women with diabetes treated with or without insulin (analogues).

Thesis outline

This thesis starts with a description of the trends in incidence rates of breast cancer in women with and without type 2 diabetes in the United Kingdom over a period of 24 years, aiming to quantify the double burden of disease (**chapter 2**). **Chapter 3** presents a quantitative and qualitative review of published *in vitro*, *in vivo* and epidemiological evidence on the postulated association between insulin and insulin analogue treatment and breast cancer development, as well as plausible mechanisms involved. In **chapter 4, 5 and 6** we show results of studies of breast tumors of women with and without diabetes. We used data and tumor tissue from primary invasive breast cancer patients that were randomly selected from an existing nationwide hospital-based cohort in Denmark. In **chapter 4** we focus on clinical-pathological characteristics of insulin and non-insulin treated women with diabetes compared to women without diabetes, and we determine whether these women develop specific breast cancer subtypes defined by clinically used IHC tumor markers (ER/PR/HER2). In **chapter 5** we investigate whether proteins within or related to the insulin signaling pathway are differentially expressed in tumors of women with or without diabetes, treated with or without insulin. Additionally, we compare protein expression between users of human insulin and insulin analogues. And finally, in **chapter 6** we study gene expression profiles of tumors of women with diabetes and specifically those who used insulin (analogues). The thesis concludes with a general discussion in **chapter 7**, in which the main findings are described and placed in perspective. Strenghts and limitations are discussed and clinical implications and suggestions for future research are given.

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Chapter 2

Trends in breast cancer incidence among women with type 2 diabetes in British general practice

Primary Care Diabetes. 2017 Feb 22. pii: S1751-9918(17)30024-4

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Summary

Aims: To quantify breast cancer incidence in women with type 2 diabetes and assess age-standardized trends in invasive breast cancer incidence over time and by age groups.

Methods: A population-based cohort study was conducted using the British general practice database (Clinical Practice Research Datalink) using data from 1989 to 2012. All adult women prescribed anti-hyperglycemic medication were selected and matched (1:1) on age and clinical practice to a reference cohort without diabetes.

Results: During approximately 1.6 million person years (py), 2,371 breast cancer cases were diagnosed in the diabetes cohort (n=147,998) and 2,252 in the reference cohort (n=147,998). Incidence of breast cancer, overall or by age groups, among women with diabetes remained stable over time. The (overall) age-standardized breast cancer IR per 100,000 py of the diabetes cohort (150, 95%CI:143-157) resembled that observed in the reference cohort (148, 95%CI:141-156); with an incidence rate ratio (IRR) of 1.01 (95%CI:0.94-1.08, p>0.05).

Conclusions: Currently, around 2,880 women with type 2 diabetes are diagnosed with breast cancer per year in the United Kingdom. However, breast cancer incidence remained stable in the last 10 years and seems to be comparable in women with and without diabetes.

Introduction

Type 2 diabetes mellitus and breast cancer are two major global health problems with partially shared risk factors such as overweight [1]. Recent estimates indicate that diabetes prevalence is 9.1% and life-time risk for breast cancer is 9.7% among women in Europe [2, 3]. Female breast cancer incidence rates (IRs) have increased strongly since the late-1970s [4], with a 62%-increase in the United Kingdom (UK) [5]. Between 2001-2012 the increases in IRs have been relatively stabilized with a total increase of ~6% [5]. For diabetes the incidence and prevalence is still rising in most European countries [6-8]. The number of women with type 2 diabetes in the UK has doubled since 1994. Age-standardized IRs of diabetes increased from 1.6 women per 1,000 person years (py) in 1994 to 3.1 women per 1,000 py in 2003 [9].

Meta-analyses have reported that women with type 2 diabetes having a 1.2-fold risk to develop breast cancer [10-14]. Changes in population lifestyle patterns over time, such as increased high-caloric diet and decreased physical activity, resulting in obesity, led to an increase in the number of people developing type 2 diabetes [15]. Possible explanations for the increased risk of breast cancer in patients with diabetes include shared risk factors such as obesity (high BMI), high blood glucose levels and hyperinsulinemia [12, 16, 17].

Ageing populations and better treatment (resulting in lower mortality rates) further contribute to the increasing prevalence of diabetes. Hence, a significant proportion of women is living with diabetes, and these women may be at increased risk of developing breast cancer. It is important for public health decisions to quantify this double burden of disease and get insight in the absolute numbers of breast cancer incidence stratified by type 2 diabetes over time. However, these numbers are largely missing. Therefore, we examined age-standardized IRs of breast cancer among women with type 2 diabetes in British general practice and investigated trends in incidence over time (1989-2012) and by age groups. To support our findings, we compared breast cancer incidence trends to a non-diabetes reference group. Since underlying risk factors changed over time we also stratified IRs by menopause (using age as a proxy) and BMI to explore whether we could identify specific subgroups of women with diabetes that might benefit from e.g. intensified breast cancer screening.

Methods

Source of data

Data were obtained from the Clinical Practice Research Datalink (CPRD) [18]. This database comprises electronic medical records from patients registered at general practices since 1987 and represents approximately 7% of the UK population. Patients in the CPRD are broadly representative of the UK general population in terms of age, sex, ethnicity, and mortality rates [18, 19]. The accuracy and

completeness of CPRD data have been well validated in previous studies [20, 21]. Data recorded in CPRD include demographic information, prescribed medication, clinical events including cancer diagnosis, preventive care provided, specialist referrals and hospital admissions. The CPRD's Independent Scientific Advisory Committee approved the protocol of this study (number: 13_050).

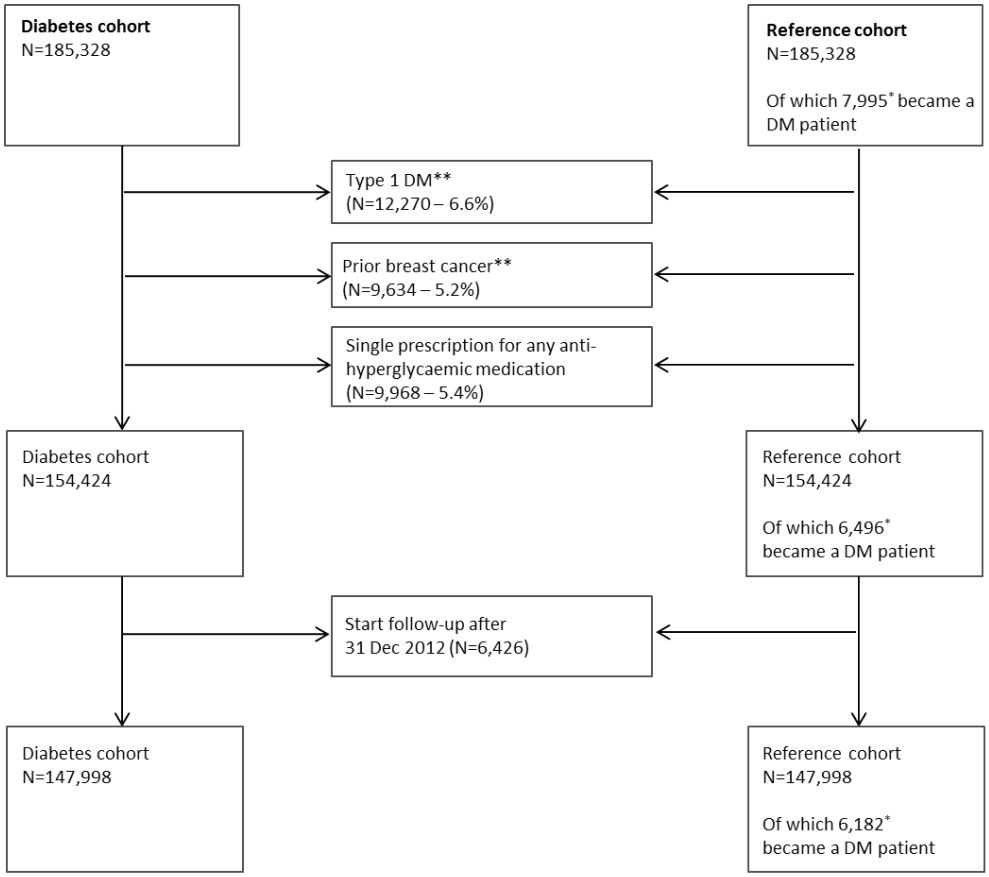
Study population, follow-up and case definition

To estimate breast cancer rates among women with and without type 2 diabetes during 1989-2012, we used a cohort of prevalent and incident anti-hyperglycemic drug users (diabetes cohort) and a matched reference cohort. The diabetes cohort consisted of registered adult women (aged ≥18 years) with at least 1 prescription for any anti-hyperglycemic agent recorded in CPRD during follow-up. The date of the first anti-hyperglycemic drug prescription during follow-up was taken as the date of cohort entry; though women might also have used anti-hyperglycemic drugs prior to cohort entry. The diabetes cohort was matched (1:1) on age and practice to a reference cohort of women without any recorded prescriptions for anti-hyperglycemic agents. If a woman in the reference cohort started using anti-hyperglycemic drugs during follow-up, she was censored and categorized as a patient with diabetes from that day onwards. As a newly diagnosed patient with diabetes, she was then matched to a new woman that was added to the reference cohort. By creating two dynamic cohorts we avoided immortal time bias [22].

To select our final cohort, we excluded patients with type-1 diabetes. Women with a prescription for insulin on the index date, without a concomitant prescription for non-insulin anti-diabetic drugs (NIADS) were considered as patients with type-1 diabetes, if (a) they had a recorded diagnosis for type-1 diabetes or (b) they were under the age of 30 at cohort entry. In addition, women with primary breast cancer prior to cohort entry, and women in the diabetes cohort without any subsequent prescription for an anti-hyperglycemic agent after the initial prescription recorded at cohort entry were excluded. If a woman with diabetes or a matched woman without diabetes met any of the exclusion criteria, the woman was excluded, together with her matched counterpart. A flowchart of the selection of the diabetes and reference cohort is presented in Figure 1.

All women were followed from cohort entry until the occurrence of breast cancer, death, transfer out of practice, or end of data collection (October 31, 2013), whichever came first. The first-ever diagnostic code for invasive breast cancer (Supplementary material Table 1) in CPRD after cohort entry was taken as the date of diagnosis. Medical records from CPRD are regarded as a valid measure to capture breast cancer occurrence [23].

Figure 1. Flow chart of the selection of the diabetes and matched reference cohorts in the CPRD (1989-2012)



* Women who were diagnosed with diabetes after attributing to the reference cohort. Follow-up was censored in the reference cohort, upon which the women with newly diagnosed diabetes was included in the diabetes cohort. A new reference patient was matched to the women with newly diagnosed diabetes. ** Several women score in multiple categories (N=968). *DM= Diabetes Mellitus*

Data analysis

For the diabetes and reference cohorts, IRs for primary invasive breast cancer were calculated and standardized for age using direct standardization by weighting all the strata according to the age distribution in the 2012 European (EU-27) standard population [24]. Confidence intervals (CI) were calculated for crude [25] and age-standardized IRs [26]. To assess secular trends, IRs are presented by calendar year period. Age categories for standardization consisted of 5-year intervals, starting with '18-20 years' and ending with '85+ years'. For calendar year period, two-year intervals were created; but 1989-2000 were aggregated due to small numbers.

In addition, we assessed IRs in age groups (<45, 45-54, 55-64, 65-69, 70-79, ≥80 years) over time, and in BMI categories (<25, ≥25 to <30, ≥30 to <35, ≥35 kg/m^2, unknown), and in pre- and postmenopausal women (age 55 years was used as proxy) over the entire follow-up period. Within the age groups we also standardized for age in 5-years intervals. Age was determined per calendar year as the year difference with the year of birth. One woman could thus contribute to different age-specific IRs in different calendar years. Rates for women <45 years over time are not presented separately as numbers were too small and we had insufficient numbers to present IRs over time stratified for BMI categories. Since menopausal status is an effect modifier in the relation between BMI and breast cancer risk, we described breast cancer incidence rates per BMI category among pre- and postmenopausal women separately. BMI was determined time-dependently, where BMI was updated with each new recording at the date of measurement. If the last measurement was older than 1 year, BMI was labeled as 'unknown'. Stratification for BMI in the reference cohort was not possible since for 76% of the women BMI was not available in the year prior to cohort entry.

Follow-up time for all women was divided in periods with variable length, depending on the occurrence of a new recording of BMI. Subsequently, IRs per BMI category were produced as the number of events within each category, divided by the total amount of follow-up time; i.e. the sum of all time periods within this category. All IRs are provided as the number of new breast cancer events per 100,000 py. Differences between IRs were determined by calculating incidence rate ratios (age-standardized IR diabetes/age-standardized IR reference) with 95% CI [26]. If this interval includes 1.0, the standardized rates are not significantly different at a 5% level. The same method was used to compare IRs in calendar year periods.

To exclude the influence of potential diagnostic bias in the comparison between women with and without diabetes (*i.e.* increased breast cancer screening around the time of initiation of diabetes treatment) [27], we performed a sensitivity analysis, in which the first year of follow-up was excluded for all women with and without diabetes. Additionally, we ran sensitivity analyses to assess whether results in pre- and postmenopausal women were similar when using age 50 as proxy for menopausal status.

Results

Characteristics of the diabetes and reference cohort

In total, 147,998 women with diabetes and 147,998 women without diabetes were included in the study with a median age of 64 years at cohort entry (Table 1). Of the women with diabetes 11% was treated with insulin and 66% with metformin at cohort entry. In the diabetes cohort, 26% of the women were obese (BMI 30-35 kg/m^2) and 31% severe obese (BMI ≥35 kg/m^2), according to the most recent measurement in the year prior to cohort entry; in the reference cohort this was 17% and 11%, respectively.

Table 1. Characteristics and number of person years of follow-up for each calendar period in the diabetes and reference cohort in the CPRD

	Diabetes cohort (n=147,998)		Reference cohort (n=147,998)	
Age in years (median, IQR)	64	(51-74)	64	(51-74)
Person years of follow-up				
Entire follow-up	805,005		777,746	
1989-2000	116,005		114,679	
2001-2002	63,347		61,437	
2003-2004	85,283		82,086	
2005-2006	106,852		102,005	
2007-2008	126,002		120,159	
2009-2010	144,473		138,833	
2011-2012	163,043		158,548	
	n	**%**	**n**	**%**
Prior cancer[a]	10,034	6.8	10,058	6.8
BMI (kg/m^2)[b]				
<20	1,578	1.9	2,804	7.9
20-25	10,627	13.1	11,487	32.3
25-30	22,321	27.5	11,439	32.2
30-35	21,398	26.3	6,050	17.0
>35	25,343	31.2	3,779	10.6
unknown	66,731	45.1	112,439	76.0
Smoking[b]				
Current	20,318	21.2	20,599	22.1
Ex	19,046	19.9	15,847	17.0
Never	56,582	59.0	56,600	60.8
Unknown	52,052	35.2	54,952	37.1
Alcohol use[b]				
Yes	49,092	63.2	54,953	74.6
No	28,645	36.8	18,697	25.4
Unknown	70,261	47.5	74,348	50.2
Type of anti-hyperglycemic drug[c]				
Insulin	15,773	10.7		
Metformin	98,259	66.4		
Sulfonylurea	45,208	30.5		
Thiazolidinediones	3,158	2.1		
Other oral anti-hyperglycemic drugs	2,251	1.5		

[a)] Any type, except non-melanoma skin cancer or breast cancer, [b)] BMI, alcohol and smoking information is based on the most recent record in the year prior to cohort entry. The denominator of the category 'unknown' is the overall number of individuals, while the percentage of sub-categories of BMI, smoking, and alcohol use is calculated relative to all those who are not 'unknown', [c)] Several patients have multiple prescriptions on the index date. Abbreviations: IQR, interquartile range; BMI, body mass-index.

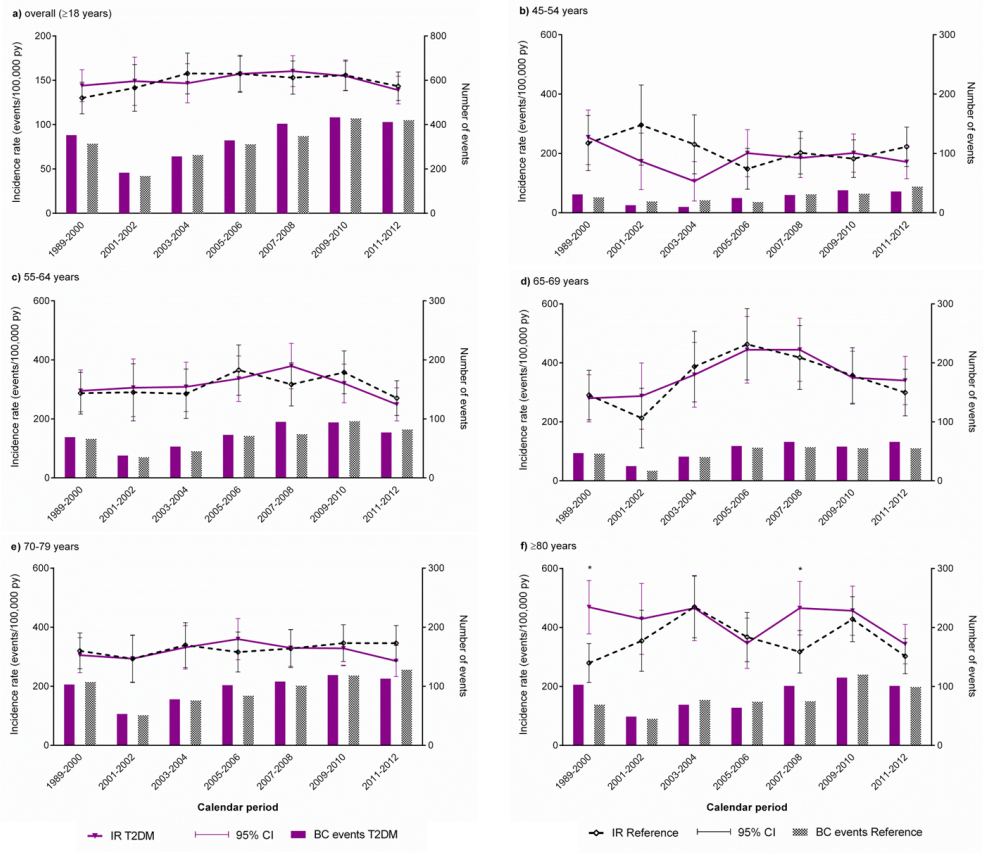
Overall incidence

During a total follow-up of approximately 1.6 million py, 2,371 women were diagnosed with invasive breast cancer in the diabetes cohort (crude IR: 295/100,000 py) and 2,252 in the reference cohort (crude IR: 290/100,000 py). Incidence of breast cancer among women with diabetes increased slightly between 1989-2008 and incidence rates declined between 2009-2012 (Figure 2a), but none of these secular trends were significant, with IRRs of respectively 1.11 (95%CI:0.94-1.31, p>0.05) and 0.87 (95%CI:0.74-1.01, p>0.05). The IRs of the diabetes cohort resembled those observed in the reference cohort over time. Overall, age-standardized breast cancer IRs per 100,000 py were similar between the diabetes (150, 95%CI:143-157) and reference cohort (148, 95%CI:141-156) with an incidence rate ratio (IRR) of 1.01 (95%CI:0.94-1.08, p>0.05). The sensitivity analysis, in which the first year of follow-up was excluded, resulted in a lower age-standardized IRs per 100,000 py for the diabetes cohort (140, 95%CI:132-148, n=141,902), but not for the reference cohort (148, 95%CI:140-157, n=141,902), with an IRR of 0.94 (95%CI:0.87-1.02, p>0.05).

Incidence by age groups

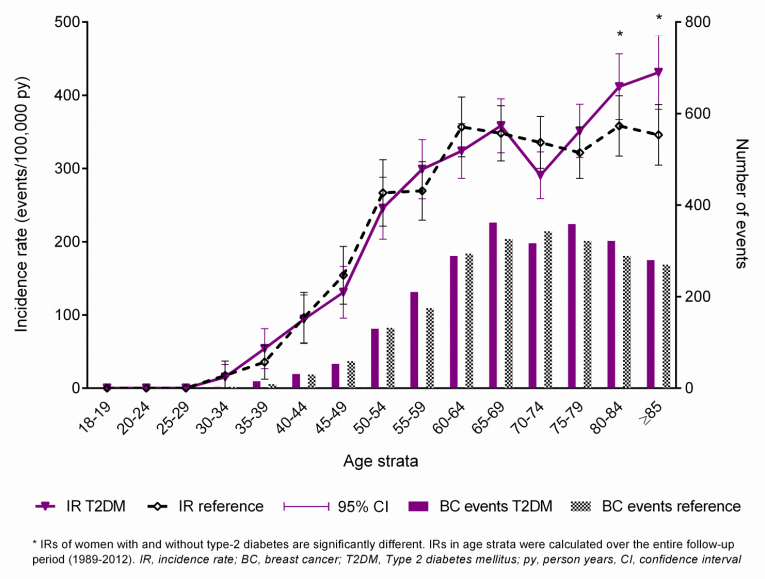
Age-specific IRs showed a constant rise by age for women with diabetes (except for a drop at age 70-74 years); the same was seen for women without diabetes but with a flattening around the age of 64 years (Figure 3). Incidence rates in women with diabetes between 80-84 years and ≥85 years were significantly higher as compared to women without diabetes; IRR 1.15 (95%CI:1.01-1.32, p<0.05) and IRR 1.25 (95%CI:1.08-1.44, p<0.05), respectively. Incidence rates per age category were reasonably stable over time (Figure 2b-f). We observed a trend of increasing IRs of breast cancer in women aged 65-69 years with significant increased incidence between 2001-2006 for women with diabetes (IRR:1.59, 95%CI:1.08-2.35, p>0.05) and without diabetes (IRR:2.18, 95%CI:1.33-3.55, p>0.05). In women with diabetes, IRs were higher in women over 80 years compared to women without diabetes, which was significant in periods 1989-2000 and 2007-2008. This is in line with the age-specific IRs presented in Figure 3.

Figure 2. Time trends in age-standardized incidence rates for breast cancer among women with and without type 2 diabetes in the CPRD (1989-2012), overall and by age group



* IRs of women with and without type-2 diabetes are significantly different; Rates are standardized for age in 5-year intervals, also within the age groups. *IR, incidence rate; BC, breast cancer; T2DM, Type-2 diabetes mellitus; py, person years; CI, confidence interval*

Figure 3. Age-specific crude incidence rates for breast cancer in women with and without type 2 diabetes in the CPRD (1989-2012)



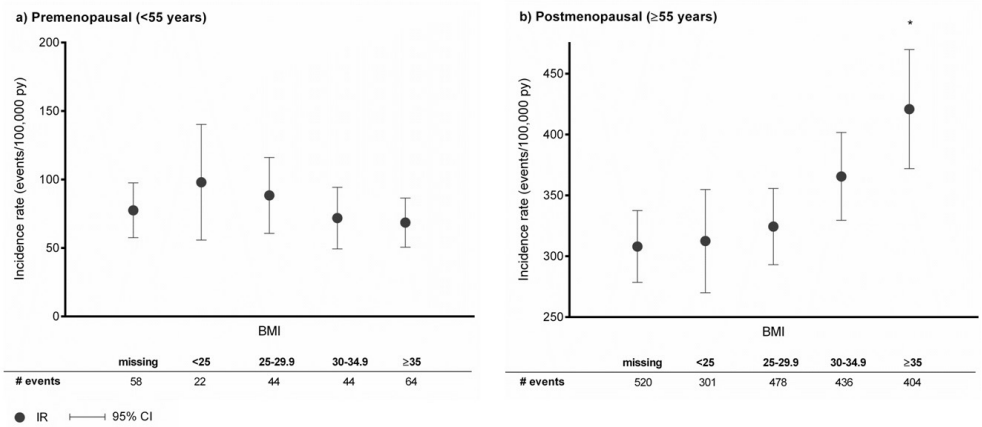
* IRs of women with and without type-2 diabetes are significantly different. IRs in age strata were calculated over the entire follow-up period (1989-2012). *IR, incidence rate; BC, breast cancer; T2DM, Type 2 diabetes mellitus; py, person years, CI, confidence interval*

Incidence by menopausal status and BMI

The observed IR in premenopausal women (<55 years) with diabetes was 77 (95%CI:67-88) and 82 (95%CI:71-93) in women without diabetes, with an IRR of 0.95 (95%CI:0.78-1.14, p>0.05). Among postmenopausal women (≥55 years) with diabetes the IR was 342 (95%CI:327-357) and the IR in women without diabetes was 330 (95%CI:315-345), with an IRR of 1.04 (95%CI:0.97-1.10, p>0.05). Sensitivity analysis, using age 50 as proxy for menopausal status gave similar results; the IRR for premenopausal women (<50 years) with diabetes compared to those without diabetes was 0.97 (95%CI:0.73-1.28, p>0.05) and for postmenopausal women (≥50 years) the IRR was 1.02 (95%CI:0.96-1.09, p>0.05).

Among premenopausal women with diabetes, age-standardized IRs of breast cancer (per 100,000 py) decreased with increasing BMI (Figure 4a), but IRRs were not significantly different (BMI≥35 vs BMI<25 kg/m^2; IRR 0.70, 95%CI:0.40-1.22). Among postmenopausal women with diabetes, age-standardized IRs of breast cancer (per 100,000 py) increased with increasing BMI (Figure 4b). Breast cancer incidence was significantly higher among postmenopausal extreme obese (BMI ≥35kg/m^2) women with diabetes compared to not-overweight (BMI<25kg/m^2) women with diabetes; IRR 1.35 (95%CI:1.13-1.61, p<0.05). The IRR for women with obesity (BMI ≥30kg/m^2) compared to not-overweight women was 1.17 (95%CI:0.99-1.38, p>0.05). Age-standardized IRs for women with diabetes with missing BMI were comparable to those with a BMI <25kg/m^2.

Figure 4. Age-standardized incidence rates for breast cancer among pre- and postmenopausal women with type 2 diabetes by BMI category in the CPRD (1989-2012)



* Significantly different compared to BMI <25. IRs in BMI categories were calculated over the entire follow-up period (1989-2012). *BMI, body mass index in kg/m2; py, person years; #, number; CI, confidence interval*

Discussion

Our study described time-trends and age-specific breast cancer IRs among women with type 2 diabetes in British general practice between 1989-2012, aiming to quantify the double burden of disease and to provide figures for public health policies. Breast cancer incidence in the diabetes cohort was similar to the reference cohort. Overall and age-specific rates of breast cancer have remained relatively stable between 2001 and 2012, apart from a temporary increase in incidence since the early 2000s among women aged 65-69 years, in both cohorts. This increase can probably to a great extent be attributed to increasing screening [28, 29], which was introduced in 1988 for women aged 50-64 years and was expanded to women aged 65-70 years in 2000.

We stratified IRs by age, menopause and BMI because of the potential modifying impact of these factors and to explore whether a subgroup of women might benefit from intensified breast cancer screening. Overall, women with and without diabetes had similar IRs by age and menopause. However, we observed that the IR of breast cancer in women >80 years was higher in women with diabetes compared to women without diabetes. Since women >80 years are not screened, it might be that breast cancer was more likely to be diagnosed due to more intensive health checks in women with diabetes.

Due to lack of completeness of BMI data, we could not make a comparison between women with and without diabetes for different BMI categories. We observed that within postmenopausal women with diabetes those with a BMI ≥35 kg/m^2 had significantly higher IRs than those not-overweight, which is in line with previous findings in women without diabetes [17]. Even though we

had indications that BMI among women with diabetes was higher than among women without diabetes in our study, we did not find an overall higher IR for breast cancer in the diabetes cohort. This might be related to lack of screening participation by obese women, possibly in particular those with diabetes [30]. Screening leads to an increase in breast cancer incidence [31], and normal weight women without diabetes are more likely to participate in screening programs [30].

Another potential modifying factor of breast cancer incidence in women with diabetes might be the use of anti-hyperglycemic agents. However, since recent published meta-analyses showed that insulin [32], as well as metformin [33], are unlikely to increase or decrease risk of breast cancer, we suspect that this, if at all, had only a minor influence on breast cancer incidence in women with diabetes.

The overall lack of difference in breast cancer incidence between the women with and without diabetes was against our expectations since previous meta-analyses of case-control and cohort studies [10, 11] showed a positive association between diabetes and breast cancer risk. Although our aim was not to perform an association study, we considered previously performed studies and compared the methodology to elaborate on this difference in outcome. Some studies included in published meta-analyses, with a large contribution to the pooled estimate, compared breast cancer risk in their cohorts of women with diabetes to IRs derived from national cancer registries [10, 11]. We estimated IRs in an age and practice-matched reference cohort of women without diabetes and we have used two dynamic cohorts to prevent immortal time bias [22]. Our design and analyses are therefore less likely to be biased than some previous studies. Another explanation for the observed discrepancies might be differences in diabetes ascertainment. We defined diabetes based on anti-hyperglycemic drug use while previous studies in the meta-analyses used hospital registries, health care databases, or questionnaires for diabetes ascertainment. Studies that included only women hospitalized for their diabetes possibly suffered from more advanced disease compared to women with diabetes in the CPRD. On the other hand, we might have missed some women with diabetes who were only treated with diet. Furthermore, the time window of observation is slightly different between our study and previous studies. Our study covers data until 2012, while previous studies ended data collection around 2000.

The Dutch Cancer Society also reported prevalence rates of diabetes among a sample of Dutch women visiting their GP and among women who were diagnosed with breast cancer [34]. They found that diabetes prevalence rates were similar among women with breast cancer (35-64 years: 3%; ≥65 years: 13.4%) compared to women without breast cancer (35-64 years: 3.1%; ≥65 years- 13.2%). These statistics are in line with our results.

If we compare our results with age-specific breast cancer IRs and time-trends in the general population published by UK cancer research [5] these were largely comparable. However, the overall age-standardized IR of our reference cohort was somewhat higher than that reported by the UK cancer registry (148 versus 125/100,000 py). This is hard to explain as 98% of the UK population is registered at a GP practice, however, the CPRD may not be representative of all

practices in the UK based on geography [18]. Underlying risk factors for breast cancer such as social status, hormone use and reproductive history might have been different between our cohort and that of the registry.

This study used a large and accurate healthcare database in which clinical records are regarded as a valid measure to capture breast cancer incidence as compared to the National Cancer Registry [23]. However, this study also had limitations. First of all, we defined diabetes based on anti-hyperglycemic drugs. Consequently, we might have missed some women with diabetes who were only treated with diet, which might have biased results toward zero. Secondly, we were unable to determine trends in incidence over time before 2001 because of the limited follow-up time and number of cancer events. However, IRs restricted to 2001 onwards were very similar to the entire follow-up period in the diabetes (151, 95%CI:143-159) and reference cohort (151, 95%CI:143-159). However, since overall incidence rates remained relatively stable over time, and the IRs were comparable between women with and without diabetes), we do not expect that these analyses would have given us new insights. Thirdly, potential diagnostic bias at the start of follow-up might be present, as the age-standardized IR for breast cancer among the diabetes cohort decreased from 150 to 140/100,000 py after elimination of the first year of follow-up. Finally, we could not match women with and without diabetes on BMI because of information asymmetry between the two cohorts. In addition, for the women without diabetes we were unable to stratify IRs for BMI categories because the majority had no recently recorded BMI measure. BMI is less frequently measured in (normal weight) women without diabetes as the Quality and Outcome Framework in the UK specifically rewards practices for the registration of BMI among patients with diabetes and among women with a BMI >30 kg/m^2 [35]. We assume that unmeasured BMI, reflects normal BMI.

The UK has approximately 1.92 million women living with diagnosed diabetes [36], of whom, assuming a similar age distribution as the women in our study and an age-standardized IR of 150/100,000 py, each year 2,880 will be diagnosed with breast cancer. This is a high number, but incidence of breast cancer among women with diabetes remained seemingly stable between 2000-2012 and breast cancer incidence in women with diabetes was comparable to incidence in women without diabetes. Therefore, based on this research there is no indication that points towards a need for a different screening approach, such as for example intensified screening for breast cancer among women with type 2 diabetes. Even so, further research is recommended in women with high BMI and diabetes since they are at higher risk and based on other studies might be less likely to attend the mammography screening.

Ethical approval and consent to participate

The CPRD's Independent Scientific Advisory Committee approved the protocol of this study (protocol no: 13_050).

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Chapter 3

Treatment with insulin (analogues) and breast cancer risk in women with diabetes; a systematic review and meta-analysis of in vitro, animal and human evidence

Breast Cancer Research 2015, 17(1):100

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Summary

Introduction: Several studies have suggested that anti-diabetic insulin analogue treatment might increase cancer risk. The aim of this study was to review the postulated association between insulin and insulin analogue treatment and breast cancer development, and plausible mechanisms.

Methods: A systematic literature search was performed on breast cell-line, animal and human studies using the key words 'insulin analogue' and 'breast neoplasia' in MEDLINE at PubMed, EMBASE, and ISI Web of Science databases. A quantitative and qualitative review was performed on the epidemiological data; due to a limited number of reported estimates, a meta-analysis was performed for glargine only. A comprehensive overview was composed for in vitro and animal studies. Protein and gene expression was analysed for the cell lines most frequently used in the included in vitro studies.

Results: In total 16 in vitro, 5 animal, 2 in vivo human and 29 epidemiological papers were included. Insulin AspB10 showed mitogenic properties in vitro and in animal studies. Glargine was the only clinically available insulin analogue for which an increased proliferative potential was found in breast cancer cell lines. However, the pooled analysis of 13 epidemiological studies did not show evidence for an association between insulin glargine treatment and an increased breast cancer risk (HR 1.04; 95% CI 0.91-1.17; p=0.49) versus no glargine in patients with diabetes mellitus. It has to be taken into account that the number of animal studies was limited, and epidemiological studies were underpowered and suffered from methodological limitations.

Conclusions: There is no compelling evidence that any clinically available insulin analogue (Aspart, Detemir, Glargine, Glulisine or Lispro), nor human insulin increases breast cancer risk. Overall, the data suggests that insulin treatment is not involved in breast tumour initiation, but might induce breast tumour progression by up regulating mitogenic signalling pathways.

Introduction

Breast cancer is the most prevalent cancer in women with 1.67 million new cancer cases diagnosed in 2012 worldwide [1]. Diabetes mellitus (DM) has been associated with breast cancer [2]. However, it is unknown if this association is due to the high blood glucose levels of DM, hyperinsulinaemia, shared risks factors such as obesity, or side-effects of diabetic treatment.

Exogenous insulin treatment for diabetics includes animal insulin, human insulin and insulin analogues. Insulin can act as a growth factor, and it is biologically plausible that use of exogenous insulin (analogues), could stimulate neoplastic growth [3]. The initial source of insulin for clinical use in humans was from animal pancreases. Gradually animal insulin has been almost completely replaced by modified or biosynthetic human insulin, such as NPH, Lente or Regular, and insulin analogues. Insulin analogues are marketed since 1997 and are different from the human insulin molecule since the amino acid sequence is modified to have an altered pharmacokinetic profile. These modifications afford greater flexibility in the treatment of diabetic patients. However, structural transformation of human insulin might also result in different binding affinity towards the IGF1 receptor. This may result in an increased mitogenic action of insulin analogues. As each insulin analogue has different alterations in their amino acid sequence, the pharmacologic properties of the analogues are slightly different. Therefore it could be that various insulin analogues have different tumour promoting properties. Glargine is theoretically most likely to have an increase mitogenic action compared to human insulin, as the carboxy terminal of the B-chain of glargine has a positive charge, as is the case with IGF-1.

In 2009, the results of four large-scale epidemiological studies were published, raising the concern that insulin analogues, especially insulin glargine, might increase risk of cancer [4-8]. Two of these studies suggested that insulin glargine may be associated with higher risk of cancer than treatment with human insulin [5, 8]. Although the results were inconsistent and the authors stressed the limitations of their studies, this led to an urgent call for more research by the European Association for the Study of Diabetes [9].

Previous reviews that focussed on in vitro studies consistently reported that glargine has, in contrast to other commercially available analogues, increased binding affinity towards Insulin-like growth factor1 receptor (IGF1R). Most studies concluded that glargine may have an increased mitogenic potential in particular at supra-physiological concentrations [10, 11]. Extrapolation of these results to humans is difficult due to obvious limitations of in vitro studies, but also due to tissue-specific biological responses. A focus on a specific cancer type could clarify this issue.

The published animal studies on insulin analogues and cancer have not been reviewed so far. In addition, meta-analyses of epidemiological studies have been inconsistent. One meta-analysis reported an increased relative risk (RR) of any cancer among insulin (analogue) users compared to non-insulin treated diabetics of 1.39 (95% Confidence Interval (CI) 1.14-1.70) [12], while another reported no effect (RR 1.04; 95% CI 0.75-1.45) [13]. Insulin use was not associated with

an increased risk of breast cancer. However, two [13, 14] out of four meta-analyses [13-16] concluded that risk of breast cancer was increased among glargine users compared to non-glargine users. Considering that cancer is a heterogeneous disease with different aetiologies involved, and breast cancer being the most common female cancer, we focussed this review on the association of exogenous insulin (analogue) exposure and the risk of breast cancer. To study breast cancer risk in an in vitro, animal and human setting, we made a distinction between tumour initiation and progression as most in vivo en in vitro studies can only address tumour progression. Furthermore, we deducted from the literature review what is currently known on signalling pathways involved in insulin-induced tumourigenesis. We included all widely prescribed insulin analogues and insulin AspB10 and included in vitro, animal, in vivo human and epidemiological studies. To our knowledge, this is the first review to provide a complete overview (including in vitro, in vivo and epidemiological evidence) on whether and how insulin analogues could affect breast cancer risk in diabetic patients.

Methods

This systematic review is registered at PROSPERO [17] with the registration number: CRD42012002477 and was developed according to the PRISMA guidelines [18], and with guidance from the Cochrane Collaboration handbook [19].

Data sources and searches

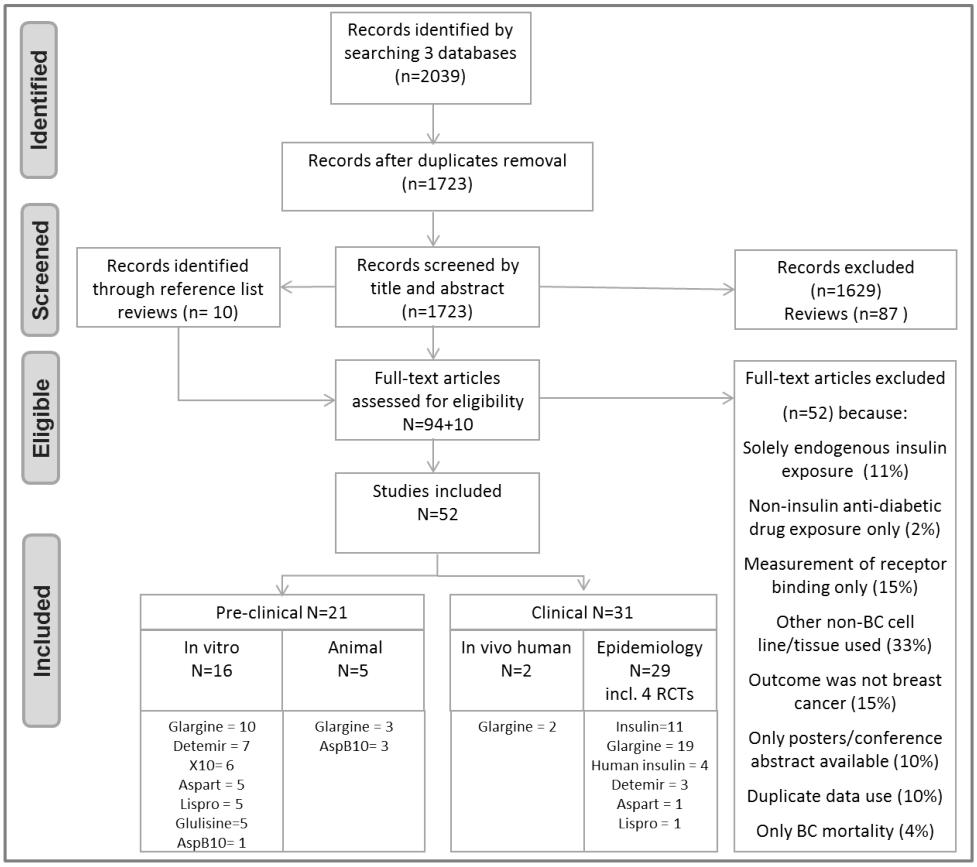
A search of MEDLINE at PubMed, EMBASE, and ISI Web of Science, was performed using key words 'insulin (analogue)' and 'breast cancer' (or similar terms) through July 2014. The full search strategy is described in the electronic supplementary material (ESM) 1.

Study selection

Eligible studies had to describe effect measures of exogenous insulin (analogue) use on breast cancer development. We included studies with direct (tumour incidence, size, volume, and metastases) or indirect outcomes (cell proliferation, count, and apoptosis, as well as genes and/or proteins explaining mechanisms of breast cancer tumour development e.g. MAPK, PI3K, PTEN, mTOR, p53) associated with breast cancer. Studies were divided in three categories with the following selection criteria; 1) in vitro studies on mammary gland cell lines exposed to insulin analogues, in which direct proliferative effect was measured or pathway activation was monitored; 2) animal studies on models treated with insulin analogue, in which the mammary gland tumour progression/initiation was measured, or different insulin analogues were compared for their activation of mitogenic signalling pathways in mammary gland tissue, and 3) epidemiological and in vivo studies in humans, including patients with type 1 or type 2 DM treated with insulin

analogues before breast cancer diagnosis; cohort and case-control studies as well as randomized controlled trials were included. Only epidemiological studies that presented relative or absolute risk estimates for breast cancer among insulin users were included. Studies that used a non-DM reference population were excluded. In case of multiple publications on the same dataset, we included the study with most complete data. An overview of the study selection is provided in Figure 1.

Figure 1. Flow chart of study identification and study selection process



Data extraction

For the in vitro and animal studies information was extracted on the cell (with INSR:IGF1R status) or animal model (species, tumour subtype), study design (in vitro: assay, starvation method, exposure time, type and refreshment of medium, and presence of phenol red; animal: tissue and proteins analysed, and time of sampling), the intervention (compounds and concentration/dose tested) and the study outcome (mammary tumour formation, mitogenic response, and pathway activation) (Tables 1 and 2).

For each epidemiological study, information was extracted on study design and characteristics, i.e. country, source population, data sources, study period, age group, matching variables for case-control studies, DM type and definition, prevalent/incident insulin users, exposure definition, time of exposure definition, mean duration of exposure, latency period, and covariates (ESM Table 2-3c); and risk estimates for each exposure comparison (Table 3).

Data synthesis and analyses

In vitro and animal studies were grouped by type of insulin analogue, and common pathways/ mechanisms of action were extracted and summarized. Plausible pathways were suggested based on the strength of the evidence. To substantiate the results of the in vitro studies included in this systematic review, we created an overview of the protein and gene expression in eight commonly used mammary (tumour) cell lines of hormone receptor levels (INSR, IGF1R, ER, PR, HER2, EGFR) and some proteins essential for insulin-induced downstream signalling cascades. The methods of these experiments can be found in ESM 2.

The exposure comparisons that were examined in the epidemiological studies were categorized as: 1) use of any exogenous insulin versus no use of any exogenous insulin (drug exposure undefined); 2) use of any exogenous insulin versus use of non-insulin anti-diabetic drug (NIAD) (type of NIAD defined); 3) use of insulin X versus no use of insulin X. Results were categorized on the exposure of interest. Data was ordered per risk estimate (Hazard Ratio (HR), Odds Ratio (OR), Incidence Rate Ratio (IRR)). If a study presented results within the same exposure comparison, but with different definitions of the exposure of interest (e.g. glargine users or glargine only users), the group that had most power was included to calculate the pooled estimate. We set a subjective cut-off of 10 studies needed for a pooled analysis; hence this was only performed for glargine. The pooled estimate was derived using the random effect model. Pooled analysis by dose or duration was not feasible, as risk estimates were reported for different exposure comparisons, exposure definitions (e.g. mean or cumulative dose, duration since start exposure, or cumulative duration) and stratification categories. The quality evaluation of the epidemiological studies focussed on potential selection bias, information bias, and confounding. In the ESM 3 the evaluation process of the bias and power of studies is displayed. Data were prepared in Microsoft Access 2010 and analysed in Stata version 11.0.

Results

A search in MEDLINE at PubMed, EMBASE, and ISI Web of Science identified 1723 unique records (Figure 1). After the eligibility assessment, 52 studies on exogenous insulin (analogue) exposure and breast cancer were included, of which 16 in vitro, 5 animal, 2 human in vivo and 29 epidemiological studies (see ESM 4 for study descriptions).

Evidence of mitogenic/carcinogenic potential

Current evidence of the mitogenic/carcinogenic potential per insulin (analogue) is described below, highlighting the most important findings displayed in the tables and figures. In Table 1 an overview is presented of all in vitro studies in which the mitogenic potency and/or stimulation of signalling pathways MAPK and PI3K upon insulin analogue(s) exposure was determined in a mammary gland (tumour) cell line [20-35]. Protein expression of hormone receptors and some downstream signalling proteins for each cell line are provided in ESM Table 1 and Figure 2. In Table 2 an overview is presented of all relevant animal studies [36-40]. Descriptions and characteristics of the epidemiological studies are presented in ESM Table 2-3c [5, 6, 41-67]. Table 3 lists the overall risk estimates for breast cancer per insulin analogue in the epidemiological studies; the corresponding forest plots are presented in ESM Figure 1. Results of the meta-analysis on glargine can be found in Figure 3. Some studies provided risk estimates by strata of duration or dose of exposure (ESM Table 4). The quality assessment of the epidemiological studies is shown in ESM Table 5.

Table 1. Overview of *in vitro* studies in breast cancer cell lines on the mitogenic potential of insulin analogues

Author, Year	Cell-line	IR : IGF1R	Method	Starvation	Stimulation time	Refreshment of medium	Type of stimulation medium	Presence phenol red	Analogues tested	Concentrations tested nM	Mitogenic response	Sig.	PI3K pathway*	MAPK pathway*
Milazzo et al, 1997[26]	MCF7[A]	1 : 4	[3H]Thymidine incorporation	Yes	24 hrs stim 2 hrs measure	Yes	MEM DME/F12 +0.1% BSA	Yes	AspB10	10	↑[A,B]	Yes		
			DNA measurement	Yes	3-5 days	Yes, every two days	MEM DME/F12 +0.1% BSA	Yes	AspB10	0.01-10	↑[A,B]	yes		
	MCF10[B]	1 : 0.8	Colony forming assay	No	2 weeks	Yes, every two days	MEM DME/F12 +2% BSA	Yes	AspB10	100	↑[A] -[B]	Yes		
Staiger et al, 2007[32]	MCF7[A]	-	[3H]Thymidine incorporation	48h[A] 24h[B]	20 hrs stim 4 hrs measure	Yes	DME/F12 SFM	No	Glargine	10, 50, 100	↓[A]	No		
	MCF10[B]	-	MTT	No	4 days	Yes, every two days	DME/F12 SFM	No	Glargine	1, 5, 10, 25	↑[A,B]	No		
Liefvendahl et al, 2008[24]	MCF7 SKBR-3	1:20 1:1.8	[3H]Thymidine incorporation	24 hrs	21 hrs stim 3 hrs measure	No	DMEM SFM	No	Glargine	0.01-100	-			
Mayer et al, 2008[25]	MCF7[A] MCF10A[B] T47D[C]	1:3 1:1.2 1:2	Cristal violet cell staining	No	4 days	No	DMEM + 1% SD-FBS	No	Aspart Lispro Glargine Glulisine Detemir	1.5[A,B] 15[A,B] 1500[C]	↑[A]	Yes[A]		
Shukla et al, 2009[31]	MCF7[A] MCF10A[B]	- -	Cristal violet cell staining	24 hrs	3 days[A] 2 days[B]	Yes, every 24 hrs	DMEM + 2% DCC-FBS[A] MEGM[B]	No	Aspart Lispro Glargine Detemir	1.5, 15, 150, 1500	↑[A] - ↑[A] ↓[A]	No yes No		
			WB	24 hrs	10 min	-	DMEM + 2% DCC-FBS[A] MEGM[B]	No	Aspart Lispro Glargine Detemir			 Yes yes	- - ↑[A,B] ↓[A]	- - ↑[A] -
Shukla et al, 2009[30]	MCF7[A] MCF10A[B]	- -	Cristal violet cell staining	24 hrs	3 days[A] 2 days[B]	Yes, every 24 hrs	DMEM + 2% DCC-FBS MEGM	No	Glulisine	1.5, 15, 150, 1500	↓[AB]	No		
			MMOC/ki67 nuclei count	No	3 days	No	Waymouth medium SFM		Glulisine	750	↓	No		
			WB	24 hrs	10 min	-	DMEM + 2% DCC-FBS[A] MEGM[B]	No	Glulisine			yYs	↓[AB]	↓[AB]

Author, Year	Cell-line	IR : IGF1R	Method	Starvation	Stimulation time	Refreshment of medium	Type of stimulation medium	Presence phenol red	Analogues tested	Concentrations tested nM	Mitogenic response	Sig.	PI3K pathway*	MAPK pathway*
Weinstein et al, 2010[35]	MCF7	-	Cell counting	No	72 hrs	Yes every day	DMEM/SFM		Glargine Detemir	100	↑ ↑	No No		
Oleksiewicz et al, 2011[27]	MCF7	-	FACS	72 hrs	24-30 hrs	No	DMEM + 0.1% FCS	No	X10	0.074-2	↑	Yes		
			WB	72 hrs	20 - 40 min	No	DMEM + 0.1% FCS	No	X10	0.67 , 2		Yes	↑	↑
Teng et al, 2011[33]	MCF7[A]	-	MTT	24 hours	2 days	Yes, every two days	RPMI + 0.5 % CS-FBS	No	Glargine	20-200	↑[A]	Yes		
												Yes		
			WB	No	0, 30, 60, 120, 240 min	No	RPMI + 0.5 % CS-FBS	No	Glargine	100nM	↑[A]			
			FACS anti-apoptotic	No	48 hrs	No	RPMI + 0.5 % CS-FBS		Glargine		↑[A] anti-Apoptotic response	Yes		
Glendorf et al, 2012[21]	HMEC	1:20	[3H]Thymidine incorporation	No	70 hrs stim 2 hrs measure	No	MEGM	?	B10A, B10R, X10, B10Q, B10E, B10H, B10I, B10F, B10W, B10V	0.0001 - 1000	↓ ↓ ↑ ↑ ↑ ↓ ↓ ↓ ↓ ↓			
Hansen et al, 2012[22]	HMEC[A]	1:21	[3H]Thymidine incorporation	24 hrs	70 hrs stim 2 hrs measure	No	MEGM	No	Detemir Glargine X10	0.001-1000	↓[A] ↑[A] ↑[A]	Yes Yes Yes		
Knudsen et al, 2012[23]	MCF7[A]	-	[3H]Thymidine incorporation	2 hrs	24 hrs stim 2 hrs measure	No	DMEM + 0.1% serum	No	S961	0.0001-100	↑[A]			
Pierre-Eugene et al, 2012[28]	MCF7[A]	-	BRET-PIP_3	No	45 min	No	DMEM/F12 + 5% FBS	?	Aspart Lispro Glargine M1 M2 Glulisine Detemir			 Yes Yes Yes	- - ↑[A] ↓[A] ↓[A] ↓[A] ↓[B]	
	MDA-MB-231[B]	-												
			WB	12	5 or 20 min	No	DMEM/F12 SFM	?	Glargine M1 M2				↑[A] - -	↑[A] - -
			[^{14}C]Thymidine incorporation	4hrs	19 hrs stim 6 hrs measure	No	DMEM/F12 SFM	?	Glargine M1 M2	0.01-1000	↑[A] - -			

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Table 1. Overview of *in vitro* studies in breast cancer cell lines on the mitogenic potential of insulin analogues (Continued)

Author, Year	Cell-line	IR : IGF1R	Method	Starvation	Stimulation time	Refreshment of medium	Type of stimulation medium	Presence phenol red	Analogues tested	Concentrations tested nM	Mitogenic response	Sig.	PI3K pathway*	MAPK pathway*
Gallagher et al, 2013[20]	MET1 MVT1		WB	1 hr	10 min	No	DMEM + 0.1% BSA		X10	10	↑	Yes		
Ter Braak et al, 2014[34]	MCF7 IGF1R[A]	1:25	WB		30 min	No	RPMI + 5% CDFBS	No	Aspart Lispro Glargine M1 M2 Glulisine Detemir X10	10, 33, 100		- ↑[A] ↑[A] - - - ↓[ABC] ↑[A]	- - ↑[ABC] - - - ↓[ABC] ↑[ABC]	
	MCF7 IRA[B]	1:0.02												
	MCF7 IRB[C]	1:0.07												
			SRB	24 hrs	4 days	Yes	RPMI + 5% CDFBS	No	Aspart Lispro Glargine M1 M2 Glulisine Detemir X10	0.01-100	- - ↑ - - - ↓ ↑	 Yes Yes Yes		
Sciacca et al, 2014[29]	MCF7[A]	1:6	BRDU incorporation	24 hrs	12 hrs, 6hrs measure	No	MEM SFM	?	Aspart Lispro Glargine M1 M2 Glulisine Detemir X10	5 nM (only detemir at 19 nM)	↓[A] –[B,C,D] –[A,C,D] ↑[B] –[A,C,D] ↑[B] –[A,B,D] ↓[C] ↑[A] –[B,D] ↓[C] –[A,C,D] ↑[B] –[A,C,D] ↑[B] ↑[A,B] –[c,D]	 Yes[B] Yes[B] Yes[B] Yes[B] Yes[B]		
	MDA-MB-157[B]	1:2												
	MDA-MB-468[C]	1:0.2												
	T47D[D]	1:8	Collagen invasion assay (Boyden chamber technique)	No	18 hrs	No	MEM SFM	?	Aspart Lispro Glargine M1 M2 Glulisine Detemir X10		–[A,D], ↑[B,C] –[A,D] ↑[B,C] ↑[A,B,C] ↓[D] ↑[A,C] –[B,D] –[A,D] ↑[B,C] ↓[A,D] ↑[B,C] ↑[A,B,C,D] ↑[A,B,C,D]			

[A/B] Often studies used multiple cell lines. In case of cell line specific conclusions the superscript A/B/C/D are used to refer to this specific cell line. * Some studies used a specific experimental setup that allowed a discrimination between the involvement of different pathways. For all these studies the p-ERK and p-AKT served as biomarker for activation of MAPK or PI3K, respectively.

Table 2. Overview of *in vivo* studies in animals on the correlation of insulin analogues and breast cancer

Author, Year	Model	Number of animals per treatment group	Tissues analysed	Time points sampling	Analogues tested	Dose tested nM	Method	Proteins analysed	Carcinogenic potential	Sig	Tumour characteristics
Stammberger et al, 2002[37] (re-evaluation in 2012)[38]	Sprague-Dawley rats and Wistar rats and NMRImice	5-30	No further tumour characterisation	Follow up of 2-years	Glargine	2, 5, 12.5 IU/Kg	Spontaneous mammary gland tumour formation upon treatment		-		MG adenoma, fibroadenoma, adenocarcinoma
Gallagher et al, 2012[36]	orthotopic mammary tumour wt and hyperinsulinaemic MKR mice	3-4	Mammary gland	0-25 days	AspB10	12.5 IU/kg 2x/day	Tumour volume measurement		↑	Yes	
			Lung metastasis				Counting Lung metastasis		↑	No	
							WB receptor activation	p-IR/ p-IGF1R	↑	Yes	
								p-Akt	↑	Yes	
								p-Erk	-		
Tennagels et al, 2013[39]	female Sprague–Dawley rats	3-4	Mammary gland	60 min	Glargine AspB10	12.5, U/kg	WB kinase activation	p-IR	-		
								p-IGF1R	↑	Yes	
Ter Braak et al, 2015[40]	p53$^{R270H/+}$WAPCre FVB mice	40	Mammary gland tumors	Chronic exposure till MG tumor development	Glargine	12.5-15 IU/kg	Tumour latency time		↑	No	majority aggressive EMT no correlation pathology and treatment
					AspB10	150-200 IU/kg			↑	Yes	
							WB protein expression profiling	IR, IGF1R, Erk, p-Erk, Akt,	↑	Yes	
								p-Akt, EGFR, ER, E-cad, N-cad, Her2	↑	Yes	

Table 3. Relative risk estimations for breast cancer among insulin treatment groups and the evaluation of bias and power of the studies

Author, Year	Exposure of interest	Exposure comparison group	Nr cases/controls*** or nr cases/person years**** in exposure group	Nr cases/controls*** or nr cases/person years****in comparison group	Risk Ratio**	95 % CI	Risk of bias	Power
Any insulin– no insulin: Hazard Ratio								
Carstensen et al, 2012[43]	Insulin users	No insulin users	248/102,500	2,118/627,100	0.96	0.84-1.09	Moderate	Adequate
Ferrara et al, 2011[48]	Insulin users	No insulin users	NR	NR	1.0	0.9-1.2	Moderate	Adequate
Neumann et al, 2012[60]	Insulin users	No insulin users	NR/NR*	NR/NR*	**0.86**	**0.81-0.91**	High	Adequate
Onitilo et al, 2014[61]	Insulin users	No insulin users	NR/NR*	NR/NR*	0.84	0.58-1.23	High	Too low
Any insulin– no insulin: Odds Ratio								
Bodmer et al, 2010a[41]	Insulin users	No insulin users	43/131	262/1,022	NE	NE	High	Too low
Cleveland et al, 2012[45]	Insulin users	No insulin users	20/16	50/49	1.15	0.40-3.40	High	Too low
Any insulin - NIAD: Hazard Ratio								
Currie et al, 2009a[6]	Insulin users	Metformin only	NR/12,640*	NR/34,847*	1.07	0.79-1.44	Moderate	Too low
Redaniel et al, 2012a[62]	Insulin and NIAD users	Sulfonylurea only users	33/8,233.8	93/27,308.2	1.23	0.63-2.38	Low	Too low
Redaniel et al, 2012b[62]	Insulin only users	Sulfonylurea only users	8/2,247.3	93/27,308.2	1.67	0.70-3.99	Low	Too low
Vallarino et al, 2013******[67]	Pioglitzone users, not using insulin	Insulin users, not using pioglitazone	181/29,721	113/13,680	0.85	0.67-1.08	High	Low
Any insulin - NIAD: Odds Ratio								
Hsieh et al, 2012[53]	Insulin only users	Metformin only users	5/NR	19/NR	1.63	0.60-4.40	High	Too low
Koro et al, 2007a[54]	Insulin and NIAD users	TZD users	13/52	83/449	0.71	0.36-1.37	High	Too low
Koro et al, 2007b[54]	Insulin only users	TZD users	9/62	83/449	1.27	0.61-2.67	High	Too low
Glargine – no glargine: Hazard Ratio								
Bordeleau et al, 2014*****[42]	Glargine users	Standard care, not using glargine	28/11,620*	28/12,845*	1.15	0.67-1.97	Low	Too low
Home and Lagarenne, 2009*****[52]	Glargine users	Any anti-diabetic drug, NPH in 20 studies	4/4,711	6/4,524	0.62	0.17-2.18	Moderate	Too low
Rosenstock et al, 2009[63]	Glargine users	NPH users	3/2,144	5/2,096	0.90	0.64-1.26	Low	Too low
Chang et al, 2011*****[44]	Glargine users, not using int-/long-acting HI	Non-glargine int/long-acting HI users	6/6,558.8*	65/47,724.6*	0.53	0.21-1.31	Moderate	Too low
Colhoun et al, 2009a[5]	Glargine plus non-glargine insulin users	Non-glargine insulin users	0/NR	29/9,667*	NE	NE	High	Too low
Colhoun et al, 2009b*****[5]	Glargine only users	Non-glargine insulin users	6/1,200*	29/9,667*	1.47	0.59-3.64	High	Too low

Author, Year	Exposure of interest	Exposure comparison group	Nr cases/controls*** or nr cases/person years**** in exposure group	Nr cases/controls*** or nr cases/person years****in comparison group	Risk Ratio**	95 % CI	Risk of bias	Power
Currie et al, 2009b*****[6]	Glargine users	Non-glargine insulin users	10/2,245*	38/8,102*	0.86	0.42-1.75	Moderate	Too low
Fagot et al, 2013a*****[47]	Glargine users	Other int-/long-acting insulin only users	114/42,129*	40/14,082*	1.08	0.72-1.62	High	Too low
Habel et al, 2013a**** *[51]	Glargine users	NPH insulin users	52/10,614.8	217/60,868.1	1.3	1.0-1.8	Moderate	Too low
Habel et al, 2013b[51]	Glargine only users	NPH insulin users	33/6,402.4	217/60,868.1	1.3	0.9-2.0	Moderate	Too low
Habel et al, 2013c[51]	Glargine and NPH insulin users	NPH insulin users	19/4,212.5	217/60,868.1	1.3	0.8-2.0	Moderate	Too low
Kostev et al, 2012a*****[55]	Glargine users	NPH insulin users	NR	NR	0.93	0.68-1.27	High	Too low
Lind et al, 2012a*****[56]	Glargine users	Non-glargine users	19/7,019.4	96/48,889.6*	1.54	0.90-2.67	Moderate	Too low
Morden et al, 2011a[59]	Glargine plus non-glargine insulin users	Non-glargine insulin users	102/18,889*	333/65,294*	1.08	0.86-1.36	High	Low
Morden et al, 2011b*****[59]	Glargine only users	Non-glargine insulin users	118/21,071*	333/65,294*	1.03	0.83-1.29	High	Low
Ruiter et al, 2012a*****[64]	Glargine only users	Human insulin only users	11/6,875*	NR; IR=2.28*	**1.65**	**1.10-2.47**	Moderate	Too low
Sturmer et al, 2013a ****[65]	Glargine users	NPH users	103/26,277	19/5,885	1.07	0.65-1.75	Moderate	Too low
Suissa et al, 2011a*****[66]	Glargine users	Non-glargine insulin users	18/6,094	60/12,262	0.8	0.3-2.1	Moderate	Too low
Pooled Hazard Ratio	*Glargine*	*No glargine*			*1.04*	*0.91-1.17*		
Glargine – no glargine: Incidence Rate Ratio								
Ljung et al, 2011a[57]	Glargine plus non-glargine insulin users	Non-glargine insulin users	59/25,033	283/101,419	1.04	0.77-1.41	High	Low
Ljung et al, 2011b[57]	Glargine only users	Non-glargine insulin users	31/7,302	283/101,419	**1.58**	**1.09-2.29**	High	Too low
Glargine – no glargine: Odds Ratio								
Grimaldi-Bensouda et al, 2013a[49]	Glargine users	Non-glargine users	78/287	697/2,763*	1.04	0.76-1.44	Low	Borderline
Grimaldi-Bensouda et al, 2013b[49]	Glargine users	Non-glargine insulin users	74/203	70/207	0.96	0.61-1.53	Low	Too low
Grimaldi-Bensouda et al, 2013c[49]	Glargine users	Human insulin users	NR	NR	1.29	0.78-2.13	Low	NE
Grimaldi-Bensouda et al, 2013d[49]	Glargine users	Aspart users	NR	NR	1.10	0.64-1.89	Low	NE
Grimaldi-Bensouda et al, 2013[e49]	Glargine users	Lispro users	NR	NR	0.85	0.48-1.50	Low	NE
Mannucci et al, 2010a[58]	Glargine users	Non-glargine insulin users	NR	NR	NE	NE	High	Too low

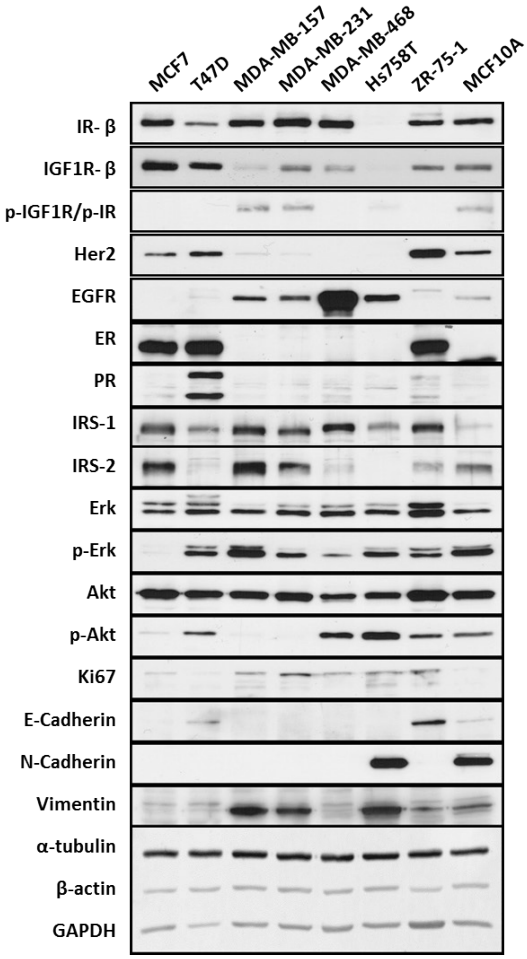
Table 3. Relative risk estimations for breast cancer among insulin treatment groups and the evaluation of bias and power of the studies (Continued)

Author, Year	Exposure of interest	Exposure comparison group	Nr cases/controls*** or nr cases/person years**** in exposure group	Nr cases/controls*** or nr cases/person years****in comparison group	Risk Ratio**	95 % CI	Risk of bias	Power
Determir – no determir: Hazard Ratio								
Fagot et al, 2013b[47]	Determir users	Other int-/long-acting insulin only users	38/12,806*	116/43,131*	1.08	0.72-1.62	High	Too low
Kostev et al, 2012b[55]	Detemir users	NPH insulin users	NR/789	NR/4,206	1.17	0.66-2.06	High	Too low
Determir – no determir: Incidence Rate Ratio								
Dejgaard et al, 2009a[46]	Determir users	NPH users	1/2,252	0/1,420	NE	NE	Low	Too low
Dejgaard et al, 2009b[46]	Determir users	Glargine users	1/917	3/628	NR	NR	Low	Too low
Aspart – no aspart: Odds Ratio								
Grimaldi-Bensouda et al, 2013f[55]	Aspart users	Non-aspart users	54/241	721/2,809*	0.95	0.64-1.40	Low	Borderline
Lispro – no lispro: Odds Ratio								
Grimaldi-Bensouda et al, 2013g[49]	Lispro users	Non-lispro users	46/133	729/2,917*	1.23	0.79-1.92	Low	Borderline
Human Insulin – no human insulin: Hazard Ratio								
Fagot et al, 2013c[47]	Basal human insulin users	Other int-/long-acting insulin only users	15/5,813*	139/50,948*	1.03	0.56-1.88	High	Too low
Gu et al, 2013[50]	Human insulin users	No insulin users	4/6,188*	14/10,435*	0.33	0.10-1.13	Moderate	Too low
Ruiter et al, 2012b[64]	Non-glargine insulin users	Human insulin only users	31/15,578*	NR; IR=2.28*	0.99	0.81-1.20	Moderate	Too low
Human Insulin – no human insulin: Odds Ratio								
Grimaldi-Bensouda et al, 2013h[49]	Human insulin users	Non-human insulin users	59/260	716/2,790*	0.81	0.55-1.20	Low	Borderline

Bold = significantly different; *Calculated using data provided (if not indicated directly taken from table in paper); **Risk estimate are adjusted for covariates as stated in supplementary table 3. Covariates used in the various analyses are the same within one study. *** Case control studies; **** Cohort studies or randomized clinical trials; ***** Included in meta-analysis; ****** The exposure of interest is the exposure comparison group in this analysis. Abbreviations: NR= not reported, NE= not estimated, HI= human insulin, TZD= Thiazolidinedione, NIAD=non-insulin anti-diabetic drug.

Studies are first ordered by type of exposure and then by type of risk estimate. Note: Hiesh 2012 is a cohort study but provided OR estimates in the paper. Names of exposure groups are defined by the authors of the study. Several papers showed multiple risk estimates for the same exposure with different analytical approaches. For each study and exposure, the results from the least biased or best performed analyses are shown; showing HRs, IRRs or ORs as applicable. Different exposure comparisons within one study are indicated by a,b,c etc. We choose to include the risk estimate that gave (in order of importance): 1) estimates for incident users was preferred over estimates for prevalent users; 2) as-treated analysis (during study period/follow up) was preferred over intention-to-treat analysis (during fixed period/at baseline); 3) estimates with, the longest, latency period were preferred. Estimates from statistical models adjusted for covariates were preferred over crude estimate.

Figure 2. Protein expression profiling of eight commonly used human breast cell lines.



Receptor levels and signalling molecules downstream of the INSR/IGF1R signalling pathway have been quantified. Furthermore some breast cancer subtype markers have been used to further characterize these cell lines that are commonly used in the research articles discussed in this review.

Insulin glargine (M1/M2)

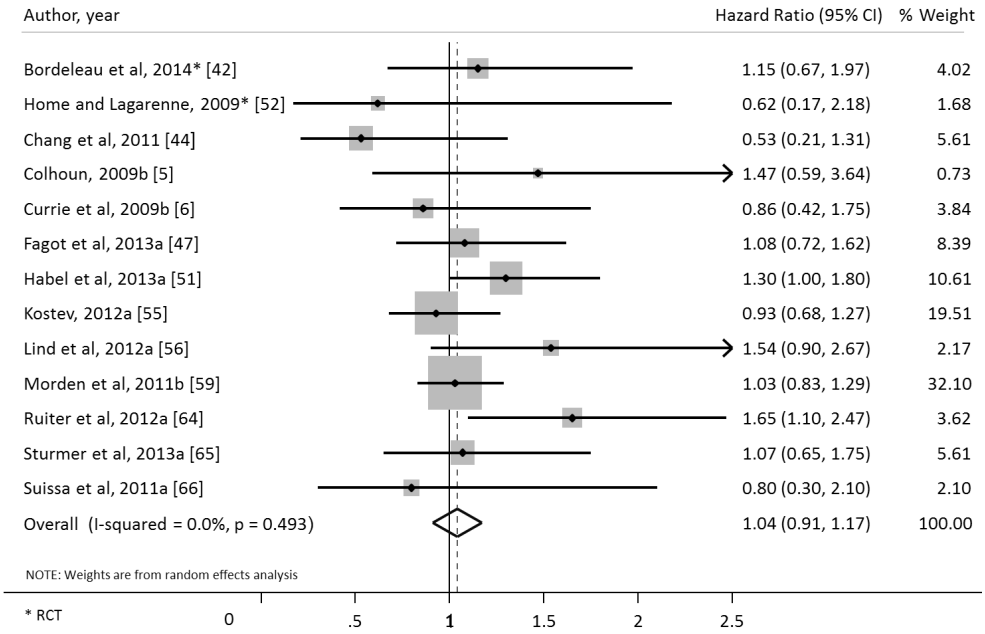
Seven of ten in vitro studies found an increased proliferative potential of glargine in comparison with human insulin [22,25,28,29,31,34,35] (Table 1). Two studies found proliferative behaviour of glargine as well, but human insulin was not included as a reference compound, therefore they could not confirm an increased proliferative response [32,33]. One study is difficult to interpret, since IGF1 did not show an increased mitogenic potential either [24]. Glargine has, similar to insulin AspB10, an increased binding affinity towards IGF1R [68]. This receptor is assumed to be responsible for the increased mitogenic action. Studies including kinase activation assays indicated that the PI3K

signalling cascade is significantly upregulated after glargine stimulation compared to human insulin stimulation [28,31,33,34]. Two studies also found the MAPK signalling cascade to be upregulated [28,31]. The clinical relevance of this increased mitogenic potential is yet unknown since glargine is rapidly metabolised in vivo into two metabolically active compounds, M1 and M2 [69,70]. These metabolites possess low mitogenic signalling [28,34].

In a 2-year follow-up study, wild type Sprague-Dawley rats, Wistar rats, and NMRI mice have been used to test the effect of chronic glargine injections compared to the insulin NPH injections; no difference in tumour free survival was observed [37,38] (Table 2). In contrast, a recent study revealed a (non-significant) decrease in tumour latency time after a similar chronic exposure to glargine; tumour multiplicity or metastases were not affected [40]. Glargine injections induced no increased receptor activation response in the mammary glands of Sprague-Dawley rats [39].

Three Randomized Clinical Trials (RCT) that investigated breast cancer risk among glargine users compared to non-glargine users [42,52,63] did not show significant differences (Table 3). Most case-control and cohort studies showed a non-significant increased risk. Only two observational studies [57,64] showed a statistically significant increased risk of breast cancer of respectively IRR 1.58 (95% CI 1.09-2.29) and HR 1.65 (95% CI 1.10-2.47). Both studies included glargine only users and compared them to non-glargine insulin users [57] and human insulin only users [64]. As the glargine studies did not show statistically significant heterogeneity (I^2=0.0%; p>0.05) a meta-analysis could be performed. The pooled HR for glargine vs. no use of glargine of 13 studies was (HR 1.04; 95% CI 0.91-1.17; p=0.49) (Figure 3 and Table 3), showing no evidence for an association between insulin glargine treatment and an increased incidence of breast cancer.

Figure 3. Forest plot reported hazard ratios for risk of breast cancer among insulin glargine users



Insulin detemir

Detemir is like glargine a long acting insulin analogue. In general, it is assumed that detemir has a lower mitogenic potential compared to human insulin [22,28,31,34], but in a number of in vitro studies a similar or even an increased proliferative behaviour was observed [25,29,35] (Table 1). The binding characteristics for detemir towards albumin are different among species. In almost all in vitro studies BSA (bovine serum albumin) or FBS (foetal bovine serum) is added to the stimulation medium. Interpretation of these mitogenicity studies is difficult since it is not yet known how the bovine albumin interacts with detemir compared to human albumin [11]. For the same reason it is not surprising that no chronic animal studies have been conducted with insulin detemir. Only three epidemiological studies have been performed, one RCT [46] and two cohort studies [47,55]; none found an association with breast cancer development (Table 3).

Insulin aspart, glulisine and lispro

Compared to glargine and detemir, the insulin analogues aspart, glulisine and lispro are less well evaluated for mitogenic potential; no increased mitogenic behaviour was found in four in vitro studies [25,28,30,34] (Table 1). Only one in vitro study suggested a small non-significant proliferative increase of aspart compared to human insulin [31]. Another in vitro study found the mitogenic potential of glulisine to be significantly lower than human insulin [30]. Evidence that lispro and glulisine had an increased proliferative potential was found in just one in vitro study and for just two of the tested cell lines (MDA-MB-157 and MDA-MB-468) [29]. We previously found that the PI3K signalling cascade is significantly more upregulated after lispro treatment than human insulin stimulation only in the IGF1R over expressing MCF7 cell line [34]. Similar as for the in vitro, epidemiological data on these short acting insulin analogues is scarce. Just one study reported ORs for aspart and lispro of 0.95 (95% CI 0.64-1.40) and 1.23 (95% CI 0.79-1.92), respectively [49] (Table 3).

Human insulin

In vitro studies showed that treatment of diabetics with human insulin has a low mitogenic potential (Table 1). From the in vivo studies it can be concluded that human insulin is not carcinogenic as the number of tumours that developed in the human insulin treated group was similar to the vehicle injected group (Table 2). Only three epidemiological studies explored the effect of human insulin, as the exposure of interest, on the risk of breast cancer. Two of these studies compared human insulin users with insulin analogue users [47,49] and found no significant difference in breast cancer risk (Table 3). The other study compared human insulin users with diabetics not treated with insulin and reported a HR of 0.33 with a relatively wide 95% CI of 0.10-1.13 [50]. This study had not enough power.

Human insulin, especially NPH users, was often used as exposure comparison group in the studies that investigated risk of breast cancer related to insulin analogue use. Most of these studies did not report significant differences in risk of breast cancer as is mentioned previously.

Insulin AspB10

The increased carcinogenic effect of insulin AspB10 was already discovered in 1992 [71]. Since then this insulin analogue has been used in many in vitro studies as a reference compound with a strong carcinogenic potential. In proliferation studies AspB10 was highly mitogenic compared to human insulin irrespective of the cell line used [21,22,26,27,29,34] (Table 1). Most studies indicated that AspB10 induces proliferation by increased IGF1R signalling, but there are indications that the INSR is also involved since increased proliferation was not fully blocked when using a specific IGF1R inhibitor [26]. One study used two murine mammary tumour cell lines, both expressing INSR and IGF1R. These cell lines were stimulated with AspB10 and only activation of IR and not IGF1R was observed [20]. In a different study it was indicated that a prolonged occupancy time of this analogue towards the INSR results in sustained activation of this receptor and subsequently increased mitogenic potency [22]. With a collagen invasion assay it was determined in several breast cancer cell lines that AspB10 has an increased invasive capacity compared to human insulin [29]. In a very elaborate kinase/inhibitor study it was found that multiple core kinases are involved in the mitogenic behaviour of AspB10 since phosphorylation of AKT, p70S6K, S6, and 4E-BP1 was found to be increased compared to human insulin exposure [27].

In animal studies, AspB10 was found to have a dose-dependent increased carcinogenic potential [71] (Table 2). Xenograft rodent models with injected mammary gland tumour cell lines were treated with either human insulin or AspB10. Tumours were significantly bigger after the AspB10 injections and, although not significant, more lung metastases were found in this treatment group. From a kinase activation analysis on these tumours a strong up regulation of p-AKT was found indicating that the carcinogenic effects of AspB10 might be a direct effect from a PI3K response [20]. A very recent study used a $p53^{R270H/+}$WAPCre mouse model, which develops spontaneous human relevant mammary gland tumours within 70 weeks, to show that chronic exposure to AspB10 significantly decreased the tumour latency time. A detailed protein expression analysis showed that tumours induced by AspB10 or IGF1 have a distinct expression pattern compared to tumours from insulin or vehicle treated mice; both the PI3K and the MAPK were found to be significantly upregulated after AspB10 and IGF1 treatment [40]. A different study focussed on the short term mitogenic effects of AspB10 and found significant stronger receptor activation in the mammary glands of Sprague-Dawley rats one hour after AspB10 injections compared to human insulin treatment [39]. As Insulin AspB10 has been shown to have mitogenic properties in in vitro and animal studies, this drug has never been available to humans.

Insulin (analogue) users versus non-insulin users or NIAD users

In the epidemiological studies, risk of breast cancer mostly showed non-significant decreased associations with insulin use versus non-insulin use (drug exposure undefined) (Table 3). These studies did not distinguish between insulin analogues and human insulin. In contrast, most studies that compared insulin users with NIAD users (irrespective of the type of NIAD used) showed non-

significant increased associations. Only one study comparing insulin users versus non-insulin users showed an statistically significant decreased breast cancer risk of HR 0.86 (95% CI 0.81-0.91) in type 2 diabetic patients [60]. However, we judge this study to be biased because the risk estimates were not adjusted for important risk factors for breast cancer and DM, immortal time bias might be present, and no data on duration of exposure was available. Exposure categories (insulin use - no insulin use and insulin use – only NIAD use) are hard to define and compare because many patients with diabetes type 2 are using insulin (analogues) simultaneous with NIADs. Most studies that are included this review investigated combined categories of exposure to insulin (analogues) and NIADs.

Dose and duration effects in epidemiological studies

No significant differences were found between strata of duration and risk of breast cancer among users of any insulin [41,43,62] and insulin glargine [49,51,56,65,66] (ESM Table 4). However, a non-significant increased risk was found after more than five years of any insulin treatment (HR 2.25; 95% CI 0.72-6.99) [62]. Among the glargine users, the study with the longest follow-up comparing exposure of four-seven years versus <four years did not observe an increased breast cancer risk [49]. Another study revealed that the risk of breast cancer increased in the first three years after start of insulin glargine use, after which the risk of breast cancer remained at the same level [56]. Results of glargine dose on the occurrence of breast cancer [47,49,56,58,59,64] showed inconsistent results (ESM Table 4). Some studies found significant increased relative risks with increasing dose [56,59,64], while others did not [47,49,58,59]; this seems partly dependent on the exposure definition. Only one of the studies investigating glargine dose used cumulative dose [47]. The results of one in vivo study in humans indicated that there is almost no glargine circulating in plasma regardless of the dose given. Plasma M1 concentration increased with increasing dose of glargine, but as was mentioned previously, M1 possesses low mitogenic signalling [70].

Discussion

Limitations of the studies and interpretation of the findings

In vitro studies

The large variation in published in vitro results can be explained by differences in study design. For example, the choice of cell line greatly affects the obtained results because the responsiveness to growth factors, like insulin and insulin analogues, may be different from one cell line to another. Based on the cell line characterization (ESM Table 1), we showed there is a striking variation in receptor expression of the human cell lines used.

Different cell lines also have different expression of the relevant receptors involved in the insulin response. The MDA-MB-231 cell line has very low expression of IGF1R. Therefore, the increased

mitogenic potential of glargine (due to enhanced IGF1R signalling) could not be detected in this cell line [28]. However, using the MCF7 cell line (which expresses very high levels of IGF1R) the increased mitogenic potential of this compound became evident [28]. Other cell lines with low or moderate expression levels of IGF1R are less suitable for a mitogenic evaluation of insulin analogues. In line with this, a recent study including four different breast cancer cell lines (MCF7, MDA-MB-157, MDA-MB-468 and T47D) found that mitogenicity of growth factors strongly depends on the cell line that was used [29]. However, the authors concluded that the INSR/IGF1R status was not the only explanatory factor. Therefore, we determined the expression of downstream signalling molecules (Figure 2). This illustrated that the poor responsiveness in the T47D and MDA-MB-468 cell lines upon glargine exposure [25,29] may be explained by low expression of IRS1 (T47D) or IRS2 (MDA-MB-468), the first downstream targets of the INSR/IGF1R.

Besides INSR/IGF1R signalling also other receptors might have a role in insulin (analogue) induced mitogenicity. Due to insulin – ER/PR crosstalk the IRS1 and subsequently the PI3K and MAPK signalling cascades can be upregulated resulting in enhanced proliferation [72]. This effect might contribute to the increased insulin (analogue) sensitivity of MCF7, T47D and ZR-75-1 compared to the triple negative cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-468 and MCF10A). Therefore, it is important to point out that primarily ER positive or triple negative breast cancer cell lines have been used in the included studies.

The majority of the mitogenicity studies used the MCF7 cell line [23-35]. It is desirable that future studies include different cell lines, so that cell line specific effects can be excluded. For translational reasons it is essential that protein expression (and especially receptor profiles) in benign human mammary gland tissues are quantified, only in that way we can determine which cell model has the highest clinical relevance.

Another important quality factor is the starvation method. For a proper effect of a specific stimulation it is essential that the target cells are deprived from other growth factors. Some studies did not starve their cells prior to the start of the assay [21,25,28,33], especially for short term assays this might have major consequences. At last, the use of proper positive and negative controls is most important for a good quality experiment. Some studies [32,33] did not include a positive control while others lack a negative control [23], thereby making it impossible to put the results in perspective. Furthermore, one study did include a positive control (IGF1) [24], but this compound did not show a positive effect, questioning the sensitivity of their experiments.

Animal studies

The type of the animal model used plays a major role in the quality of animal studies. Generally, it is thought that rats are more sensitive in terms of carcinogenicity towards compounds and have a higher clinical relevance than mouse models [73]. But there are also major disadvantages, like higher costs and the lack of good humanized breast cancer rat models. Two studies that used rats have rather small group sizes, which obviously affected the power of their studies [37-39]. The

doses that were used in the reviewed animal studies are quite comparable to each other and are all thought to be supra-physiological (i.e. over 50 times the human dose, based on nmol/kg). In one study a non-equimolar comparison was made between the different compounds, but doses had been chosen to induce an equi-pharmacological/metabolic response [40]. In another study a high mortality was observed, probably due to hypoglycaemia, therefore the dose was lowered in a later phase of this study [39]. Surprisingly, other studies that used similar doses did not observe hypoglycaemia [37,38,40]. To verify the sensitivity of the models and techniques it is essential that the appropriate controls are included. Half of the included animal studies lacked proper controls. In our opinion both insulin and IGF1 (and ideally also AspB10) should always serve as controls to be able to put the obtained results in to perspective.

Epidemiological studies

The epidemiological studies included in this review have many limitations and results are difficult to compare across studies because the exposure of interest and exposure comparison groups have been defined differently. For example, some studies compared glargine only users with human insulin only users [64], while others compared glargine users with non-glargine insulin users [66]. In this case, the comparator is a mix of several exposures, which may affect the conclusion about the effect of a certain insulin (analogue). Some studies examined several definitions for the exposure of interest and indeed this resulted in slightly different effect estimates [57,59]. Moreover, it is difficult to disentangle the effect of insulin and the role of NIADs because most diabetics treated with insulin, have prescriptions of NIADs as well. However, it is important to do so, because some studies have shown anti-tumour effects of metformin, the most prescribed NIAD among type 2 diabetics [74]. Of note, the quality of some of these metformin studies is doubtful as well.

Inclusion criteria differed largely among studies. For example, some studies included patients with only one insulin prescription while others included continuous users over a period of six months. More important, there was large variation in the time of exposure definition. Some studies determined the use of different insulin types at baseline or during a fixed period (intention to treat), while others determined insulin exposure during follow-up (time-dependently). This may lead to patients with only one specific insulin prescription during follow-up being falsely classified as continuous users during the whole period. Cumulative exposure over time, censoring for discontinuation, or switching and latency period could affect the results. The uncertainty surrounding the extent to which a registered prescription dispensed for an insulin analogue reflects real life use of insulin analogues limits the ability to detect the true effect on the occurrence of breast cancer. Furthermore, studies variably included incident and prevalent users of insulin compromising estimates of association between the duration of use and breast cancer development.

Other methodological aspects that are important when interpreting the results of these studies are: incorrect and too short exposure time (max 3.8 years mean exposure time), reverse causation,

confounding by indication, and residual confounding (ESM 3). Most studies were based on type 2 DM, and/or did not specify type of DM. Risk of bias was classified as low (for definition see ESM 3) in only five studies [42,46,49,62,63], but in these studies power was not adequate (ESM Table 5). Of these studies, only two studies considered breast cancer as a main outcome [49,62]. Most risk estimates have wide CIs, due to lack of power of the study. Two of the three studies that found significant different results were classified as having a high risk of bias [57,60] or even so had lack of power [57,64]. So far there is not a single very well-designed study that investigated insulin treatment and breast cancer risk as main outcome, and had sufficient power. The included RCTs had limitations too, such as limited follow-up (except for one RCT with a follow-up of six years [42]), insufficient power, or cancer incidence as a secondary outcome [63,75].

All layers of evidence in perspective

Studies in humans are the gold standard for evaluating evidence of exposure and disease. The epidemiological studies reviewed varied in study design and exposure definition to a too large extent among different insulin analogues to evaluate their impact on breast cancer risk estimates. The risk estimates seemed not to be biased by important confounders as adjusted and unadjusted risk estimates only differed slightly. However, unmeasured confounding may still be present. In addition, the upper limit of the 95% CI of the pooled risk estimate of BC among glargine users was 1.17. This strengthens our idea that if any, the risk increase of breast cancer due to currently used insulin (analogues) is likely to be very small.

A distinction should be made between studying tumour initiation or progression, though in the human setting it difficult to discern these because of potential lag time in detection of cancer. The epidemiological studies investigated incidence of primary breast tumours upon insulin treatment in DM patients. True tumour initiation in animal studies can only be investigated with long-term exposure in rodents, which are costly experiments. The animal xenograft models and in vitro studies mammary tumour cell line summarized here investigated tumour progression; e.g. by evaluation of cell proliferation or up regulation of mitogenic pathways. All together, the results of this systematic review suggest that insulin treatment might be involved in tumour promotion.

Another issue to be raised is that breast cancer is not one disease but consists of different subtypes, e.g. Estrogen Receptor (ER) positive or negative, with different prognosis. The promotion of tumour cell growth upon insulin exposure may differ for different breast cancer subtypes. However, there is very limited human/epidemiological data from only two studies on the association of tumour subtypes and insulin (analogues) exposure among diabetic breast cancer patients [49,76]. More data is available about the prognosis of diabetics with breast cancer. It has been shown that overall mortality after breast cancer diagnosis is 50% higher in diabetic women compared to their non-diabetic counterparts [45,62,77,78], even after adjustment for stage [77,78]. However, whether this increased mortality is breast cancer-related or caused by comorbidities related to DM is not clear. Breast cancer in patients with DM is often diagnosed at an advanced

stage compared to patients without DM [77-80]. But studies that investigated the association between breast cancer-specific mortality and diabetes show inconsistent results [45,78,80,81]. Among patients with type 2 DM, insulin treatment was associated with a worse cancer outcome and increased all-cause mortality compared to metformin treatment [78, 82]. Only one study investigated the effect of cumulative dose and duration of insulin treatment on breast cancer specific survival, and found lower breast cancer mortality [83].

Conclusion

Based on the current epidemiological and animal data there is no compelling evidence that any clinically available insulin analogue, or human insulin increases breast cancer risk. However, animal data was limited and there is not a single very well-designed epidemiological study that investigated insulin treatment and breast cancer risk as main outcome and had sufficient power. Large randomized clinical trials were negative for increased breast cancer risk for glargine, but longer follow-up may be needed to detect delayed or smaller effects. In vitro studies have shown that only insulin AspB10 and glargine have an increased mitogenic potential compared to regular human insulin in breast cancer cell lines. The relevance of this finding for the clinical situation is unknown since AspB10 is not used in humans and it has been shown that glargine is rapidly metabolized in vivo into M1 and M2, metabolites with a low mitogenic potential. Evidence on the potential pathways involved in insulin analogue-induced breast cancer mitogenesis is limited.

Unanswered questions and future research

Except for Insulin AspB10, which has never been available to humans, all insulin analogues are still marketed. Although, there is evidence from in vitro data that insulin glargine has an increased mitogenic potential, so far, epidemiological studies have not shown evidence for an association between insulin (analogue) treatment and breast cancer risk in female diabetic patients. However, due to relatively short follow-up time in the epidemiological studies, it cannot be excluded that diabetic patients with pre-neoplastic lesions might be at higher risk of developing an invasive tumour when given a specific insulin treatment. Research on this topic is important but is still largely lacking. Therefore, we are awaiting the results of on-going efforts to pool multiple large national databases from different countries to perform a retrospective observational study in humans with a proper design, enough patients and long follow-up. Additionally, further research in the aetiology of insulin and breast cancer development is important.

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Supplementary material

ESM 1. Search strategy for each database, study selection and results

Search strategy and study selection

Online literatures searches have been updated up to July 28th 2014. Subject headings and Mesh terms were used for the search depending on the database used. We also searched in references lists of the identified reviews for papers we missed. There were no restrictions on publication date or publication status. Articles in Dutch or English were included. Two reviewers (HKB, BtB), developed and performed the search strategy for each database; duplicate references were removed (figure 1). Both reviewers independently screened title and abstract of the records for inclusion. BtB assessed the full text records of in vitro and animal studies, HKB of epidemiological and cohort studies for inclusion in the review. Reasons for exclusion were discussed.

Search terms

Web of Science

TS=("insulin analo*" OR "insulin derivative*" OR "insulin homolo*" OR glargine OR LANTUS OR degludec OR tresiba OR NPH OR lispro OR humalog OR detemir OR levemir OR glulisine OR apidra OR aspart OR novolog OR AspB10 OR X10 OR "insulin treatment" OR "diabetes treatment" OR "insulin therapy" OR "diabetes therapy") AND TS=("mammary gland" OR "breast neoplas*" OR "mammary tumor" OR "mammary cancer" OR "breast cancer " OR "breast carcinoma" OR malignan* OR carcinog* OR mitoge*)

# of articles: 587

Medline (PubMed)

(((("Insulin analogue" OR "insulin analogues" OR "insulin analog" OR "insulin analogs" OR "insulin derivative" OR "insulin derivatives" OR "insulin homologue" OR "insulin homologues" OR glargine OR LANTUS OR degludec OR tresiba OR NPH OR lispro OR humalog OR detemir OR levemir OR glulisine OR apidra OR aspart OR novolog OR AspB10 OR X10 OR "insulin treatment" OR "diabetes treatment" OR "insulin therapy" OR "diabetes therapy")[Title/Abstract]) OR "Insulin/analogs and derivatives"[MeSH]) AND (("mammary gland" OR "breast neoplasia" OR "mammary tumor" OR "mammary cancer" OR "breast cancer" OR "breast carcinoma" OR malignancy OR carcinogen OR carcinogenic OR mitogen OR mitogenic[Title/Abstract]) OR "Breast Neoplasms"[MeSH]))

# of articles: 1212

Embase

Insulin derivative/ or insulin aspart/ or insulin aspart plus insulin degludec/ or insulin degludec/ or insulin detemir/ or insulin glargine/ or insulin glulisine/ or insulin lispro/ or long acting insulin/ or short acting insulin/ AND breast cancer/ or breast tumor/ or breast carcinogenesis/

# of articles: 240

ESM 2. Characterization of cells lines

Cell line selection and culturing

Cell lines that were studied in the *in vitro* experiments are; MCF7, T47D, MDA-MB-157, MDA-MB-231, MDA-MB-468, Hs578T, ZR-75-1 and MCF10A. These cell lines are often used in other *in vitro* studies included in this systematic review. All cell lines were obtained from ATCC (Manassas, VA, USA) and were kindly provided to us by John A. Foekens and John W.M. Martens (Erasmus University Medical Center, Rotterdam, The Netherlands).

Cells were seeded in a 6-well format at a confluence of 60% in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-steptomycin (Invitrogen). Plates were incubated for 30 hours at 37˚C and 5% CO_2 followed by cell lysis.

Antibodies and reagents

Antibodies against rabbit anti-phospho-IGF1Rβ (tyr1135/1136)/phospho-IRβ (Tyr1150/1151), anti-Akt, anti-phospho-Akt (Ser473), anti-Erk, anti-phospho-Erk (Thr202,Tyr204), anti-HER2 (Cell Signaling Technology, Danvers, MA, USA), mouse anti-IGF1Rβ, anti-β-Actin, anti-GAPDH and rabbit anti-IRβ, anti-EGFR, anti-ER-α, anti-IRS-1, anti-IRS-2, (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-α-tubulin and rat anti-E-cadherin (Sigma-aldrich, St. Louis, MO, USA) and mouse anti-N-cadherin (BD translaboratories) were commercially purchased. Conjugated secondary antibodies included anti-mouse horseradish peroxidase (HRP), anti-rabbit HRP, anti-rat HRP, anti-goat HRP and Cy-5 conjugated anti-mouse have been purchased from Jackson (Jackson ImmunoResearch, West Grove, PA, USA).

Western blot analysis

The cell lysis, protein quantification and western blot analysis was performed as previously described by Li et al [1]. 40 µg of total protein was loaded per lane. For the tubulin, Actin and GAPDH blots, Cy-5 conjugated secondary antibodies were used which were visualized using a Typhoon 9400 imager. HRP conjugated secondary antibodies have been used for all the other proteins. These blots have been exposed to Pierce® ECL Western blotting substrate (Thermo Scientific, Rockford, IL, USA). Proteins were visualized by bringing the membranes in contact with an X-ray film (GE Healthcare, Little Chalfont, England). The film was developed with a Kodak X-omat 1000 processor. All bands have been quantified using ImageJ software (ImageJ, 1.43u). To correct for loading perturbations all bands have been divided by the tubulin levels of that specific blot. ZR-75-1 cell line showed basal protein expression levels of all of the receptors. Therefore, the protein expression levels of all receptors have been normalized against the levels of ZR-75-1.

Gene expression analysis

For the gene expression analysis we a used RNA normalized micro-array data from the Sanger Institute (http://cancer.sanger.ac.uk/cell_lines/download). This dataset has ArrayExpress accession number E-MTAB-3610. In a gene wise manner we expressed these values as fold changes compared to the expression levels of ZR-75-1, as we did for the protein expression analysis.

ESM 3. Method for quality evaluation of epidemiological studies

After definition of the criteria, the epidemiological studies were evaluated for study quality by two reviewers (HKB, OK). Studies differ in methodological aspects. We focused on potential selection bias, information bias, confounding bias and lack of power on the basis of information presented in the publications. Risk of bias is summarized in low, moderate and high based on a (subjective) qualitative evaluation of selection, information and confounding bias (ESM 11). These variables that were used to determine risk of bias and lack of power are presented in the ESM7-9 and table 3 respectively.

Selection bias: For the follow-up studies we first evaluated the selection of the index and control groups. We evaluated at baseline whether the cancer risk was already substantially different in both groups in a way the adjustment for difference in prognosis is not possible. Secondly, we evaluated loss-to-follow-up, especially evaluating whether the loss-to-follow-up was different in the index and control group and related to cancer/survival risk. Within the case-control studies we evaluated selection bias by evaluating whether the cases and controls came from the same population. If cases were not matched to controls on calendar time and potential exposure time, we considered if time window bias could be present.

Information bias: To evaluate whether exposure could have been misclassified we determined if exposure was measure cumulative over time, if investigators censored for switching or discontinuation of insulin treatment and whether a latency time was included. The variables data source exposure, time of exposure definition, the duration of exposure to insulin, prevalent/incident user and latency period were used to determine the above mentioned criteria. If studies did not include a latency period this could have led to breast cancer diagnosis, which was not due to the exposure of interest. This might have resulted in misclassification of the exposure-outcome relation. Studies with an intention to treat approach were indicative for risk of bias, as it assumes that the effects of exposure would continue beyond the exposure period. For the studies that reported the cumulative exposure, immortal time bias was considered. Immortal time bias was apparent if follow-up (py/exposure of interest) includes unexposed time. Unknown exposure time before cohort entry in prevalent user cohort, was considered to lead to information bias as well. It is known that one prescription of insulin is a good predictor for actual insulin analogue use of a diabetic patient. This have been proven for patients with diabetes type 1 [2], therefore we did

not take exposure definition (minimum number of prescriptions to be defined as exposed) into account in this quality evaluation.

Confounding: To evaluate the potential bias due to confounding factors, we evaluated whether the effect estimations were matched or adjusted for the following variables: age, BMI, DM duration, other DM medication than medication of interest and physical activity. Also important risk factors for breast cancer were taken into account, like family history of BC, parity, age at menarche, age at first birth, menopausal status, HRT use and anti-contraceptive pill use. All variables that were not adjusted for are listed in ESM11.

Lack of power: The number of exposed patients to be studied to identify a relative breast cancer risk of 1.2 with 80% power, α=0.05 was calculated for cohort and case control. Cut off values of the minimum required number of exposed patients were used to evaluate if the studies included in the review had enough power. In addition, the number of breast cancer cases were taken into account, e.g. if a study includes a large population but follow-up is short, the number of cases can still be small. For the cohort studies power was calculated using the methods described by Rothman [3] and Miettinen [4]. Cumulative breast cancer incidence over 10 years in Europe was calculated to estimate the risk in the unexposed patients (incidence rate per 100,000: 94.2) [5]. It was assumed that the ratio of unexposed versus exposed patients was 2:1 respectively. Based on these numbers our estimation was that the total required number of patients exposed to the insulin analogue of interest was 35,000 and 70,000 patients exposed to the reference compound. For case-control studies power was calculated using Power and Sample Size Program version 3.1.2. It was assumed that 1 cases was matched to 4 matched controls and that the probability of exposure to insulin among controls was 0.55%. Studies were powered to detect an OR of at least 1.2 based on recruitment of 1000 cases and 4000 controls.

Besides the type of bias that are included in the quality evaluation of the studies, other aspects are also important to take into account while interpreting the results of these studies. These methodological aspects have not been discusses per study, as some of these are applicable for most of the studies. First of all, incorrect definition of exposure time can lead to information bias. The longest duration of cumulative exposure was 3.5 years, while carcinogens have long latency periods. Secondly, studies may suffer from reverse causality. It might be due to subclinical phase of breast cancer that the need for insulin treatment changes and therefore it seems that insulin causes cancer while actually this is affected by the undetected breast cancer itself. Thirdly, studies may suffer from confounding by indication; subjects who use insulin are more likely to developing breast cancer due to other factors. Breast cancer incidence might differ between different diabetic medications even if the medication itself has not such an effect. There might also be systematic differences in characteristics between treatment groups. All cohort studies, except for one [6] were

not matched on patient characteristics, which results in a lack of comparability and most likely residual confounding. Additionally, some studies included patients with DM1 and DM2. Most studies that only included DM2 patients, derived DM type based on the age at onset and cut offs were different across the studies. Furthermore, it is hard to distinguish between the role of diabetes itself in the potential carcinogenic effect and the role of insulin analogues. This might have biased the results. Randomized controlled trials are free of confounding (by indication), but the trials that were included [7-10] had other limitations, such as short follow-up, a lack of power and in 2 of the studies, the outcome of interest was a secondary objective. Therefore we cannot compare these results.

ESM 4. Description of the included studies

In vitro studies

Study characteristics of the in vitro studies are summarized in table 1. Seven different human breast cancer cell lines and one immortalized cell line were used. Protein expression of hormone receptors INSR, IGF1R, ER, PR, HER2 and EGFR and some downstream signalling proteins for each cell line are provided in figure 3 and table 2.

A total of 14 different assays are described. These assays have different readouts and therefore the conclusions that can be drawn are different. Proliferation assays (MTT, [H]Thymidine incorporation, Brdu incorporation, SRB, DNA measurement, Cristal violet cell staining, ki67 or Cell counting) will shed light on the direct mitogenic potential of the compounds, whereas with functional assays (colony forming assay, collagen invasion assay, Western blotting, FACS or Bret-PIP3)) a more specific question can be addressed (e.g. ability to invade or the involvement of a particular protein in a specific process).The experimental procedures varied significantly as well, e.g. the exposure time ranged from 5 min to 5 days.

Animal studies

Descriptions of the animal studies can be found in table 2. The number of relevant animal studies was very limited and the set-up varied largely.

Human studies

Four randomized clinical trials (RCT), 5 case-control studies (2 nested case-control studies) and 20 cohort studies were included. Twelve studies investigated the effect of any exposure to exogenous insulin on the incidence of breast cancer; Nineteen studies investigated different types of insulin analogues. For most insulin analogues very few studies were published, except for long acting insulin glargine (figure 1). Descriptions and characteristics of these studies are presented in ESM 6-9.

The status and definition of diabetes, and variables that relate to insulin exposure vary among studies. Seventeen studies restricted the study population to patients with DMT2 only, though

the majority of patient in the other studies were also DMT2. Fifteen studies included only incident insulin users, i.e., patients who received their first insulin prescription during the study period. Total follow-up ranged from 1.9 to 7.1 years, and mean duration of glargine treatment ranged from 0.9 to 3.5 years. Latency periods varied from 3 to 36 months.

Only two in vivo studies in humans have been performed. One study determined plasma levels of insulin glargine and its metabolites M1 and M2 after glargine injection in patients with type 1 DM. The other study investigated clinical and breast tumour characteristics of patients with diabetes treated with glargine or other insulin analogues.

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Supplementary tables

ESM Table 1. Protein and gene expression of hormone receptors for *in vitro* human mammary cell lines included

Cell line	Origin/type of cells	IR		IGF1R		IR:IGF1R ratio		ER		PR		HER2		EGFR	
		P.E.	G.E.	P.E.	G.E.	P.E.	G.E.	P.E.	G.E.	P.E.	G.E.	P.E.	G.E.	P.E.	G.E.
MCF7	Adenocarcinoma/epithelial	1.19	0.76	7.48	2.41	1: 6.3	1:3.2	1.71	1.15	0.11	8.31	0.04	0.11	0.20	0.99
T47D	Ductal Carcinoma/Epithelial	0.47	0.38	2.43	1.23	1: 5.2	1:3.2	1.03	0.80	10.68	12.11	0.54	0.13	0.30	1.08
MDA-MB-157	Medulallary carcinoma/Epithelial	1.19	1.20	0.11	0.77	1: 0.1	1:0.6	0.00	0.12	0.02	0.00	0.02	0.12	10.73	1.03
MDA-MB-231	Adenocarcinoma/Epithelial	1.58	0.61	0.97	0.20	1: 0.6	1:0.3	0.00	0.00	0.03	0.08	0.01	0.00	10.59	1.50
MDA-MB-468	Adenocarcinoma/Epithelial	1.54	0.76	0.48	0.00	1: 0.3	NA	0.00	0.03	0.03	0.43	0.00	0.03	53.06	2.61
Hs578T	Carcinoma/Fibroblast	0.00	0.00	0.01	1.16	1: 4.7	NA	0.00	0.04	0.02	0.16	0.00	0.02	20.29	1.38
ZR-75-1	Ductal Carcinoma/Epithelial	1.00	1.00	1.00	1.00	1: 1.0	1:1.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MCF10A	Mammary gland (benign) /Epithelial	1.22		1.48		1: 1.2		0.00		0.01		0.43		4.56	

Abbreviations: P.E.= the quantified protein expression levels based on the Westernblot analysis (Figure 2). G.E.= the quantified gene expression levels of the corresponding cell lines based on the Micro Array data of the Sanger Institute.

ESM Table 2. Description of epidemiological studies included in the systematic review

Author, Year, Country of study	Study design	Source population	Data source population	Diabetes type and definition	Indicator for the exposure comparison ***	Exposure comparison (n)**	Exposure comparison (n)**	Age
						Exposure group	Reference	
Bodmer et al, 2010 [41] UK	Nested case-control	Nationwide	General Practice Research Database (GPRD)	T2DM: diagnosed > 30 years	a	Insulin users (43/131)	No insulin users (262/1,022)	30-79
Cleveland et al, 2012 [45] USA	Case-control	Population (Nassau and Suffolk counties of Long Island)	Long Island Breast Cancer Study Project (LIBCSP)	T2DM: diagnosed ≥ 30 years		Insulin users (20/16)	No insulin users (50/49)	Mean: DM 63.6 non-DM 57.4
Grimaldi-Bensouda et al, 2013 [49] UK, Canada, France	Case-control	France: nationwide UK: England,Scotland Canada: Quebec, Ontario, and New-Brunswick	Oncology clinics (medical records)	T1DM/T2DM; NR	a	Glargine users (78/287)	Non-glargine users (697/2,763*)	≥ 18
					b	Glargine users (74/203)	Non-glargine insulin users (70/207)	
					c	Glargine users (NR)	Human insulin users (NR)	
					d	Glargine users (NR)	Aspartat users (NR)	
					e	Glargine users (NR)	Lispro users (NR)	
					f	Aspart users (54/241)	Non-aspart users (721/2,809*)	
					g	Lispro users (46/133)	Non-lispro users (729/2,917*)	
					h	Human insulin users (59/260)	Non-human insulin users (716/2,790*)	
Koro et al, 2007 [54] USA	Nested case-control	NR (covers 9 census region; 30 different healthcare plans; 38 million patients)	Insurance database (Integrated Healthcare Information Services (IHCIS)	T2DM: ICD-9 code 250.x.	a	Insulin and NIAD users (13/52)	TZD users (83/449)	≥ 18
					b	Insulin only users (9/62)	TZD users (83/449)	
Mannucci et al, 2010 [58] Italy	Nested case-control	Florence	Diabetes cohort	T2DM; clinical diagnoses	a	Glargine users (NR)	Non-glargine insulin users (NR)	Mean: cases 68.9 ± 9.9, controls 68.0 ± 10.0
Carstensen et al, 2012 [43] Denmark	Cohort	Nationwide	Diabetes register (National Danish Diabetes Register)	T2DM: diagnosed 35 years		Insulin users (NR)	No insulin users (NR)	all

Author, Year, Country of study	Study design	Source population	Data source population	Diabetes type and definition	Indicator for the exposure comparison ***	Exposure comparison (n)**	Exposure comparison (n)**	Age
						Exposure group	Reference	
Chang et al, 2011 [44] Taiwan	Cohort	Nationwide	Insurance database (Taiwan's National Health Insurance (TNHI) claims database)	T2DM; T1DM excluded: ICD-9 code 250.x1 or 250.x3		Glargine users, not using int-/long-acting HI (4,566)	Non-glargine int/ long-acting HI users (23,377)	≥ 18
Colhoun et al, 2009 [5] Scotland	Cohort	Nationwide	Clinical diabetes database (Scottish Care Information-Diabetes Collaboration (SCI-DC))	T2DM: diagnosed ≥ 35 years	a	Glargine plus non-glargine insulin users (NR)	Non-glargine insulin users (NR)	adults
					b	Glargine only users (NR)	Non-glargine insulin users (NR)	
Currie et al, 2009 [6] UK	Cohort	Nationwide	General practice database (The Health Information Network (THIN))	T2DM: diagnoses > 40 years	a	Insulin users (4,432)	Metformin only (13,834)	Mean: 63.7 ± 12.9
					b	Glargine users (959)	Non-glargine insulin users (2,314)	
Fagot et al, 2013 [47] France	Cohort	Nationwide	Insurance database (French National Health Insurance Information system (SNIIRAM))	T2DM: ≥3 NIAD prescriptions in calendar year before exposure to insulin	a	Glargine users (25,298)	Other int-/long-acting insulin only users (8,687)	40-79
					b	Detemir users (8,302)	Other int-/long-acting insulin only users (25,683)	
					c	Basal human insulin users (3,401)	Other int-/long-acting insulin only users (30,584)	
Ferrara et al, 2011 [48] USA	Cohort	Northern California	Diabetes register (Kaiser Permanente Northern California Diabetes Registry (KPNC))	NR; diabetes related records from several sources		Insulin users (51,511)	No insulin users (200,956)	≥ 40
Gu et al, 2013 [50] China	Cohort	Shanghai	Shanghai Diabetes Register (SDR) database	T2DM; NR (from DM register)		Human insulin users (1,765)	No insulin users (2,340)	> 30
Habel et al, 2013 [51] USA	Cohort	Northern and Southern California	Health plan register (Kaiser Permanente Northern and Southern California (KPNC and KPSC))	T1DM/T2DM; Diabetes related records from several sources	a	Glargine users (2,869)	NPH insulin users (19,591)	≥ 18

ESM Table 2. Description of epidemiological studies included in the systematic review (Continued)

Author, Year, Country of study	Study design	Source population	Data source population	Diabetes type and definition	Indicator for the exposure comparison ***	Exposure comparison (n)**	Exposure comparison (n)**	Age
						Exposure group	Reference	
					b	Glargine only users (NR)	NPH insulin users (19,591)	
					c	Glargine and NPH insulin users (NR)	NPH insulin users (19,591)	
Hsieh et al, 2012 [53] Taiwan	Cohort	Random sample of nationwide database	Insurance database (Taiwan's National Health Insurance (NHI) claims database)	T2DM: ICD-9 code 250.x0 or 250.x2		Insulin only users (338)	Metformin only users (2,048)	Mean: 61.4 ± 13.2
Kostev, 2012 [55] Germany (Letter)	Cohort	NR (covers 20 million patients in Germany)	Research database with data from general practitioners and clinical specialists (IMS Disease Analyzer)	T2DM; NR	a	Glargine users (4,727)	NPH insulin users (4,206)	Mean: 67.5 ± 11.2
					b	Detemir users (789)	NPH insulin users (4,206)	
Lind et al, 2012 [56] Sweden	Cohort	NR (17 hospitals in Sweden)	Clinical diabetes database (Diab-base)	T1DM(42%)/T2DM/ unspecified; NR (from DM register)	a	Glargine users (2,014)	Non-glargine users (5,928)	13-97
Ljung et al, 2011 [57] Sweden	Cohort	Nationwide	Prescription database (combination of Prescribed Drug Register	T1DM: diagnosed < 30 years and T2DM: diagnosed > 30 years	a	Glargine plus non-glargine insulin users (8.889)	Non-glargine insulin users (38,152)	35-84
					b	Glargine only users (2,697)	Non-glargine insulin users (38,152)	
Morden et al, 2011 [59] USA	Cohort	Nationwide	Insurance database (Medicare)	T2DM; ICD-9 code 250.x0 or 250.x2	a	Glargine plus non-glargine insulin users (10,375)	Non-glargine insulin users (34,789)	≥ 68
					b	Glargine only users (10,857)	Non-glargine insulin users (34,789)	
Neumann et al, 2012 [60] France	Cohort	Nationwide	Insurance database (French National Health Insurance Information system (SNIIRAM)	T2DM: NIAD prescriptions in calendar year before exposure to insulin		Insulin users (179,618*)	No insulin users (491,892*)	40-79
Onitilo et al, 2014 [61] USA	Cohort	North-central Wisconsin	Marshfield Clinic electronic medical records (EMR)	T2DM; ICD-9 code 250.x0 or 250.x2		Insulin users (1,377*)	No insulin users, hba1c >7% (3,153*)	≥ 30

Author, Year, Country of study	Study design	Source population	Data source population	Diabetes type and definition	Indicator for the exposure comparison ***	Exposure comparison (n)**	Exposure comparison (n)**	Age
						Exposure group	Reference	
Redaniel et al, 2012 [62] UK	Cohort	Nationwide	General Practise Research Database (GPRD)	T2DM: diagnosed ≥ 35 years	a	Insulin and NIAD users (2,127)	Sulfonylurea only users (4,815)	> 35
					b	Insulin only users (434)	Sulfonylurea only users (4,815)	
Ruiter et al, 2012 [64] Netherlands	Cohort	Pharmo database from community pharmacies in the Netherlands (covers 2.5 million individuals)	Prescription database (PHARMO)	T2DM; T1DM excluded: patient using only insulin	a	Glargine only users (1,888)	Human insulin only users (5,093)	≥ 18
					b	Non-glargine insulin users (3,101)	Human insulin only users (5,093)	
Sturmer et al, 2013 [65] USA	Cohort	NR, US citizens enrolled in a health plan (covers >76 million inidividuals; 295 000 physicians; 185 000 clinical facilities)	Health plan registry (Inovalon Medical Outcomes Research for Effectiveness and Economics Registry (MORE))	T1DM/T2DM; ICD-9 code 250.xx	a	Glargine users (22,936)	NPH users (5,536)	≥ 18
Suissa et al, 2011 [66] UK	Matched cohort ****	Nationwide	General Practise Research Database (GPRD)	T2DM: diagnosed ≥ 40 years	a	Glargine users (1,604)	Non-glargine insulin users (3,086)	≥ 40
Vallarino et al, 2013 [67] USA	Cohort	NR, US citizens enrolled a healthcare insurance plans (covers 47 million individuals)	United healthcare insurance plan database (i3 InVision Data Mart)	T2DM; ICD-9 code 250.x0 or 250.x2		Pioglitzone users, not using insulin (15,589)	Insulin users, not using pioglitazone (8,444)	≥ 45
Bordeleau et al, 2014 [42] Canada	RCT	International multicentre study (40 countries)	Clinical sites participating in the ORIGIN trial	Impaired fasting glucose, impaired glucose tolerance, early T2DM; clinical diagnosis		Glargine users (6,264)	Standard care, not using glargine (6,273)	≥ 50
Dejgaard et al, 2009 [46] Denmark	Several RCTs	NR, participants of 21 Novo Nordisk-sponsored RCTs	Individual patient data (IPD) from Novo Nordisk sponsored trials	T1DM (9 studies)/T2DM (11 studies); NR	a	Detemir users (3,983)	NPH users (2,661)	adults
					b	Detemir users (1,219)	Glargine users (830)	
Home and Lagarenne 2009 [52] UK, USA	Several RCTs	NR, participants of 31 RCTs registered at sanofis-aventis	Pharmacovigilance database (sanofi-aventis)	T1DM (12 studies)/T2DM (19 studies); NR		Glargine users (5,657)	Any anti-diabetic drug, NPH in 20 studies (5,223)	all
Rosenstock et al, 2009 [63] USA, Canada	RCT	Multicentre study in USA and Canada	Medical centres participating in RCT	T2DM; diagnosed for ≥ 1 year		Glargine users (514)	NPH users (503)	30-70

*Calculated using data provided (if not indicated directly taken from table in paper), ** In case-control studies, n reflects cases/controls, *** Different exposure comparisons within one study are indicated by a,b,c etc., **** Matched on birth year; calender time. Abbreviations: NR= not reported, T1DM= type 1 diabetes mellitus, T2DM= type 2 diabetes mellitus, BC= breast cancer, NIAD=non-insulin anti-diabetic

ESM Table 3a. Characteristics of the case control studies included in the systematic review

Author, Year	Study period	Data source controls*	Matching variables*	Data source exposure**	Data source outcome**	Exposure definition ****	Time of exposure definition**	Duration of exposure prior to index date**	Latency period**	Exclusion of patients with a history of cancer	Covariates***
Bodmer et al, 2010 [41]	1994-2005	General Practice Research Database (GPRD)	Age; general practise; index date	General Practice Research Database (GPRD); prescribed	General Practice Research Database (GPRD)	≥ 1 oral drug prescription	During study period: in mutually exclusive treatment groups	mean: NR, # prescriptions in categories (Sup T4)	0	Yes, any cancer	BMI; hba1c; DM duration; smoking; acarbose; oestrogen; other DM medication ******
Cleveland et al, 2012 [45]	Life time exposure, BC diagnosis 1996-1997	Long Island Breast Cancer Study Project (LIBCSP). Controls were female residents from Nassau and Suffolk	Frequency matched by 5-years age groups	Interview	Physician and medical records	≥ 3 months consecutive treatment with ADD	Recalling past diabetes medication by a interview at study inclusion	NR	0	Yes, breast cancer	BMI; menopausal status; race; other DM medication ******
Grimaldi-Bensouda et al, 2013 [49]	2000-2009	General practitioners network; Pharmaco-epidemiologic General Research eXtension program (medical records)	Age; type of DM; country/ region; date of recruitment; referral to diabetologist	Interview, validated by prescriptions from GP records	Pathology and computerized oncology records	≥ 3 months treatment with insulin	During study period: for each ADD exposure (yes/no) was defined	Mean for glargine (years): 3.2 ± 2.0 in whole study population	3	Yes, breast cancer	Age; BMI; DM duration; breast cancer risk score (many variables); comorbidities; annual number of physician visits; oral ADD use; past-insulin use; other insulin use; other medication use
Koro et al, 2007 [54]	1997-2004	Insurance database (IHCIS)	Age; sex; index date; length of follow-up in the database	Insurance database (IHCIS); claims	Insurance database (IHCIS)	≥ 1 ADD prescription	During study period: for each ADD exposure (ever/never) was defined. Mutually exclusive treatment groups were made	NR	0	Yes, breast cancer	Age
Mannucci et al, 2010***** [58]	1998-2008	Diabetes cohort	Age; sex; BMI; length of follow-up	Clinical records; prescriptions	Hospital admission (Regional Hospital Discharge System) or death register (Mortality register of Tuscany)	≥ 1 insulin prescription	During study period: for each insulin type duration and mean daily dose of treatment was calculated	Median for glargine (years): 1.67 (0.8-2.3) in cases, 1.2 (0.4-2.2) in controls	12	Yes, any cancer	Comorbidity; metformin; total insulin dose; dose per insulin type; proportion of subjects with MDD ≥0.3 IU/kg/day per insulin type

* used to evaluate potential selection bias, ** used to evaluate potential information bias, *** used to evaluate potential confounding bias, **** minimum number of prescriptions during a specified period, ***** incident users, ****** other covariates were assessed but not included in the final model as they had no impact on the risk estimate. Abbreviations: NR= not reported, DM= diabetes mellitus, BC= breast cancer, ADD= anti-diabetic drugs

ESM Table 3b. Characteristics of the cohort studies included in the systematic review

Author, Year	Study period	Data source exposure**	Data source outcome**	Prevalent/ incident user**	Exposure definition ****	Time of exposure definition**	Mean duration of exposure (years)**	Latency period**	Exclusion of patients with a history of cancer	Covariates***
Carstensen et al, 2012 [43]	1995-2009	Diabetes register or prescription database	Danisch Cancer Registry	Incident	≥ 2 insulin prescriptions	During follow-up (intervals): exposure status and duration were updated	NR	1	Yes, any cancer	Age; date of birth; sex; calendar time
Chang et al, 2011 [44]	2004-2007	Insurance database (TNHI); claims	Insurance database (TNHI)	Incident	≥ 1 insulin prescription	During follow-up: exclusive users during whole follow-up period	Glargine: 1.4 HI: 2.0	0	Yes, any cancer	Age; DM-related complications; comorbidities; health service utilization; outpatient visits diabetes and non-diabetes; physician characteristics; statins; aspirin; initiation year insulin; dose of fast-acting insulin
Colhoun et al, 2009 [5]	2002/3-2005	Clinical diabetes database (SCI-DC))	Cancer register (Scottish Morbidity Record) and death register (General Registrar's Office for Scotland)	Incident	≥ 1 insulin prescription during 4 months period	During fixed period (4 months), follow-up starts after this period	NR	4	No; exclusion of patients with prior cancer did not affect the risk estimate	Age; calendar year; prior cancer; DM type
Currie et al, 2009 [6]	2000-?	General practice database (THIN)	General practice database (THIN)	Incident	≥ 1 insulin prescriptions	During follow-up: exposure status changes when a new drug of interest is prescribed.		6	Yes, any cancer	Age; sex; smoking status; diagnosis of a prior cancer *****
Fagot et al, 2013 [47]	2007-2010	Insurance database (SNIIRAM); reimbursements	Hospital discharge database (Programme de Medicalisation des Systemes d'Information (PMSI))	Incident	≥ 2 prescriptions of the same insulin type during 6 months period	At baseline, first prescription of an insulin type. Not censored if discontinued or switched	Median Glargine: 2.67 Detemir: 2.75 HI: 2.83	12	Yes, any cancer	NIAD class; DM duration *****

ESM Table 3b. Characteristics of the cohort studies included in the systematic review (Continued)

Author, Year	Study period	Data source exposure**	Data source outcome**	Prevalent/ incident user**	Exposure definition ****	Time of exposure definition**	Mean duration of exposure (years)**	Latency period**	Exclusion of patients with a history of cancer	Covariates***
Ferrara et al, 2011 [48]	1997-2005	Pharmacy database (dispensed)	Cancer registry of KPNC	Incident	≥ 2 prescriptions of the same ADD during 6 months period (ever user)	During follow-up: ever use (yes/no) changes over time	NR	6	Yes, any cancer	Age; year cohort entry; hba1c; DM duration; new DM diagnosis; smoking; ethnicity; income; creatinine; congestive heart failure; other DM medication
Gu et al, 2013 [50]	2001-2011	Diabetes register (SDR)	Shanghai Municipal Center for Disease Control and Prevention	Incident	≥ 6 months treatment with ADD	During follow-up: insulin use (yes/no)	Any human insulin: 3.37 No insulin: 4.23	0	Yes, any cancer	Age; hba1c; DM duration; smoking status; macrovascular disease; concomitant NIAD
Habel et al, 2013 [51]	2001-2009	Computerized outpatient pharmacy records; dispensed	Cancer registry of KPNC and KPSC	Incident	≥ 2 prescription of the same insulin type during 6 months period	During follow-up: ever use (yes/no) changes over time	Median Glargine: 1.2 NPH: 1.4 (full cohort)	0	Yes, any cancer	Age; site; year of entry; metformin; insulin *****
Hsieh et al, 2012 [53]	2002-2008	Insurance database (NHI); claims	Insurance database (NHI)	Prevalent	≥ 1 insulin prescription	During follow-up: exclusive users during whole follow-up period	NR	0	Yes, any cancer	Age
Kostev et al, 2012 [55]	2000-2011	Research database (IMS disease analyzer); prescribed	NR	Prevalent	≥ 1 insulin prescription	NR	NR	0	NR	Age; sex; hba1c; cumulative duration of exposure; private insurance status; urban location of practise; region; Charlson Comobidity Index
Lind et al, 2012 [56]	1985-2007	Clinical diabetes database (Diab-base)	Cancer registry and cause of death register	NR	≥ 1 insulin prescription	During follow-up (intervals): exposure status and duration were updated	Glargine: 3.5 Non-glargine: NR	0	Yes, breast cancer	Age; BMI; DM type; time since start follow-up; time since start glargine; last insulin dose used; smoking

Author, Year	Study period	Data source exposure**	Data source outcome**	Prevalent/ incident user**	Exposure definition ****	Time of exposure definition**	Mean duration of exposure (years)**	Latency period**	Exclusion of patients with a history of cancer	Covariates***
Ljung et al, 2011 [57]	2006-2008	Pharmacy database (Swedish Prescribed Drug Register); dispensed	Cancer register and Cause of death register	Prevalent	≥ 1 insulin prescription during 6 months period	During fixed period (6 months), follow-up starts after this period	NR	6	Yes, breast cancer	Age; BMI; age at onset of diabetes; smoking; age at first child birth; oestrogen; cardiovascular disease
Morden et al, 2011 [59]	2006-2008	Insurance database; claims	Insurance database	Prevalent	≥ 1 insulin prescription during 4 months period	During fixed period (4 months) mutually exclusive groups were defined. Follow-up starts after this period	Glargine only: 1.9 Non-glargine insulin: 1.9	4	Yes, breast cancer	Age; obesity; smoking; insulin dose; metformin use; ethnicity; DM complications; estrogen; poverty; 14 Charlson comorbidities
Neumann et al, 2012 [60]	2006-2009	Insurance database (SNIIRAM); reimbursements	Hospital discharge database (Programme de Medicalisation des Systemes d'Information (PMSI))	Prevalent	≥ 2 prescription of insulin during 6 months period	During follow-up: insulin use (yes/no)	NR	6	Yes, breast and bladder cancer	Age; NIAD
Onitilo et al, 2014 [61]	1995-2011	Medical records (EMR)	Medical records (EMR) and cancer registry	Incident	≥ 1 ADD prescription	Time dependent follow-up: drug use (yes/no) changes over time	NR	0	Yes, breast and colon cancer	Age; BMI; date of DM diagnosis; hba1c; comorbidities; smoking history; insurance status; location of residence
Redaniel et al, 2012 [62]	1987-2007	General Practice Research Database (GPRD); prescribed	General Practice Research Database (GPRD)	Incident	≥ 6 months of continuous prescriptions for the same ADD class (insulin; sulfonylurea; metformin; other NIAD)	Time dependent follow-up: type and duration of drug use is determined over time, including drug overlap and prescription gaps. Treatment patterns were identified (mutually exclusive groups) by chronological order of drug prescriptions	NR	36; 0 in strata of duration	Yes, breast cancer	Age; BMI; period; region; year of diagnosed. In analysis stratified by duration of exposure: + weighted hba1c

ESM Table 3b. Characteristics of the cohort studies included in the systematic review (Continued)

Author, Year	Study period	Data source exposure**	Data source outcome**	Prevalent/ incident user**	Exposure definition ****	Time of exposure definition**	Mean duration of exposure (years)**	Latency period**	Exclusion of patients with a history of cancer	Covariates***
Ruiter et al, 2012 [64]	2000-2008	Pharmacy database; dispensed	Hospital discharge records database (Dutch National Medical Register)	Incident	≥ 1 ADD prescription	During follow-up: duration of exposure. Exposure categories were mutually exclusive	Mean duration follow-up since 1st prescription Glargine: 2.2 Other insulin: 3.2 HI: 3.8	12	Yes, any cancer	Age; sex; calender time; other insulin types than exposure and comparison; nr of unique drugs used and nr of hospitalisations in the year prior to insulin start; prior NIAD use in days
Sturmer et al, 2013 [65]	2003-2010	Dispensed prescription medication claims captured in the MORE registry	Health plan register (MORE)	Incident	≥ 2 prescription of the same insulin type during 6 months period	At baseline, censored if discontinued or switched	Median Glargine: 0.9 NPH: 0.8	12	Yes, any cancer	Age; year of cohort entry; medications; comorbidities; hospitalizations; days in hospital; physician encounters; ED visits; screening tests
Suissa et al, 2011 [66]	2002-2009	General Practice Research Database (GPRD); prescribed	General Practice Research Database (GPRD)	Incident	≥ 1 insulin prescription	At baseline, not censored if discontinued or switched	NR	12	Yes, breast cancer	Age; obesity; hba1c; DM duration; excessive alcohol use; smoking status; oophorectomy; history of cancer; use of HRT; statins; other DM medication
Vallarino et al, 2013 [67]	2003-2010	Drug prescription claims captured in the i3 database	Health plan register (i3 database)	Incident	≥ 2 prescription for either pioglitazone or insulin during 6 months period	During follow-up: exclusive users (yes/no) during whole follow-up period	NR	6	Yes, any cancer	Inverse probability of treatment weights, i.e. propensity score (age, calendar year index date, obesity, medical conditions, NIAD, other medications)

*Not reported, therefore calculated by person years/n, ** used to evaluate potential information bias, *** used to evaluate potential confounding bias, **** minimum number of prescriptions during a specified period, ***** other covariates were assessed but not included in the final model as they had no impact on the risk estimate. Abbreviations: NR= not reported, DM= diabetes mellitus, HI= human insulin, ADD= anti-diabetic drugs, NIAD= non-insulin anti-diabetic drugs, HR= hazard ratio

ESM Table 3c. Characteristics of the randomized clinical trials included in the systematic review

Author, Year	Study period	Data source exposure **	Data source outcome**	Prevalent/ incident user**	Exposure definition****	Mean duration of exposure (years)**	Latency period**	Exclusion of patients with a history of cancer	Covariates***
Bordeleau et al, 2014 [42]	2003-2011	RCT database	Cancer requiring hospitalization was collected and patients were asked retrospectively about cancer not requiring hospitalisation.	Prevalent	Glargine arm: glargine once daily, standard care arm: treated on the basis of the investigators best judgement	Trial of 6 years Glargine and standard care: mean: 5.6*, median: 6.2. Mean glargine adherence was 87.1%, in the standard care group 11% used non-glargine insulin	36	Patients with an expected survival <3 years are excluded	Treatment allocation at randomization; DM status at baseline; previous CV disease status; smoking; use of metformin and sulfonylurea. Age, DM duration, BMI, prior NIAD use and fasting plasma glucose were similar between treatment arms.
Dejgaard et al, 2009 [46]	NA, different per RCT	IPD (Novo Nordisk)	Adverse event databases from each RCT	NR	Detemir arm; glargine or NPH as comparator arm	Detemir vs. NPH trial median: 0.46 years; Detemir vs. glargine median: 0.98 year	NR	NR	Age, DM status, DM duration, BMI and HbA1c were similar between the treatment arms
Home and Lagarenne 2009 [52]	NA, different per RCT	Pharmacovigilance database (sanofi-aventis)	Pharmacovigilance database (sanofi-aventis)	NR	glargine arm and 'any anti-diabetic drug' arm	Most studies: 0.5 years Glargine 0.8* Any anti-diabetic drug 0.9*	NR	NR	NR, different per RCT
Rosenstock et al, 2009 [63]	2001-2007	RCT database	Adverse events were reported by the investigator, as routine safety monitoring	Prevalent	glargine arm: glargine once daily, NPH arm: NPH twice daily	Trial of 5 years Glargine and NPH: 4.2* Prior exposure any insulin (% exposed; duration in years) glargine group: 67%, 5.5 NPH group: 70%, 4.9	0	No	NR; Age, DM status, DM duration, BMI, NIAD duration, prior insulin use, HbA1c and fasting plasma glucose were similar between the treatment arms

* Not reported, therefore calculated by person years/n, ** used to evaluate potential information bias, *** used to evaluate potential confounding bias, **** minimum number of prescriptions during a specified period. Abbreviations: NR= not reported, NA= not applicable, DM= diabetes mellitus, NIAD= non-insulin anti-diabetic drugs

ESM Table 4. Relative risk estimations for breast cancer among different duration and dose categories within insulin treatment groups

Author year	Exposure	Comparator	Breast cancer (n) exposure	Breast cancer (n) comparator	Definition of duration	Definition of dose	Category	Risk estimate*	95 % CI
Duration									
Insulin - NIAD: Hazard Ratio									
Redaniel et al, 2012c [62]	Insulin only users	Sulfonylurea only users	NR per category, 8 total	NR per category, 93 total	Duration since start exposure		<1 year	1.01	0.11-8.97
							1-5 years	0.54	0.18-1.68
							>5 years	2.25	0.72-6.99
Insulin - no insulin: Odds Ratio									
Bodmer et al, 2010 [41]	Insulin users	No insulin users	18	262	# of prescriptions, >40 reflects an exposure over 5 years		1-9	1.74	0.95-3.21
			11	262			10-29	1.30	0.62-2.70
			14	262			>29	1.51	0.76-3.01
Glargine – no glargine: Hazard Ratio									
Habel et al, 2013c [51]	Glargine only users	NPH insulin users	22	217	Duration since start exposure. Duration was calculated by adding the days between prescriptions		<2 years	1.2	0.7-1.9
			11	217			≥2 years	1.7	0.9-3.2
Lind et al, 2012b [56]	Glargine users	Non-glargine users	19	96	Hazard function of time since start of glargine		Per year	1.18	0.84-1.67
Sturmer et al, 2013b [65]	Glargine users	NPH users	37	7	Duration of drug use started from the second prescription until a patient stopped using the drugs or filled a prescription for another long-acting insulin		<6 months	0.99	0.46-2.13
			29	3			6-11 months	1.50	0.52-4.31
			26	6			12-23 months	1.09	0.38-3.12
			11	3			≥24 months	0.67	0.18-2.54
Suissa et al, 2011b [66]	Glargine users	Non-glargine insulin users	6	16	Duration since start exposure		<1 year	1.0	0.3-3.1
			8	23			1-3 years	0.9	0.3-2.7
			4	14			3-5 years	0.8	0.2-3.1
			0	7			>5 years	NE	NE
Glargine – no glargine: Odds Ratio									
Grimaldi-Bensouda et al, 2013i [49]	Glargine users	Non-glargine users	NR	NR	Total duration of each insulin. The period of use was computed based on start/stop dates and switching		<4 years	1.15	0.70-1.88
							4-7 years	0.94	0.51-1.74

Author year	Exposure	Comparator	Breast cancer (n) exposure	Breast cancer (n) comparator	Definition of duration	Definition of dose	Category	Risk estimate*	95 % CI
Dose									
Glargine – no glargine: Hazard Ratio									
Fagot et al, 2013g [47]	Glargine users	Other int-/ long-acting insulin only users	NR per category, 114 total	NR		Cumulative dose based on first insulin prescribed. Calculated by evenly distributing total dose of each insulin prescription over the days between the prescription date and the subsequent prescription date	<14000 IU	0.88	0.54-1.45
							14000-27000 IU	1.02	0.62-1.67
							>27000 IU	1.49	0.91-2.45
Lind et al, 2012c [56]	Glargine users	Non-glargine users	19	96		Hazard function of dose of glargine per Unit	Per unit	1.01	1.00-1.02
Morden et al, 2011c [59]	Glargine plus non-glargine insulin users	Non-glargine insulin users	NR	NR		Patients with mean daily dose in highest quartile	Highest quartile: 119 IU/day	1.00	0.57-1.76
Morden et al, 2011d [59]	Glargine only users	Non-glargine insulin users	NR	NR		Patients with mean daily dose in highest quartile	Highest quartile: 119 IU/day	1.75	1.10-2.78
Ruiter et al, 2012c [64]	Glargine only users	Human insulin only users	2	NR		Stratified for median dose of first insulin prescription	<median dose	NE	NE
			15	NR			=median dose	1.22	0.91-1.64
			11	NR			>median dose	2.81	1.23-6.44
Glargine – no glargine: Odds Ratio									
Grimaldi-Bensouda et al, 2013j [49]	Glargine users	Non-glargine users	31	70		Classified as < or > than median value for each insulin type in controls	Low dose: <27 dose	1.10	0.61-1.97
			33	70			High dose: >27 IU	1.02	0.59-1.75
Mannucci et al, 2010 [58]	Proportion of case subjects using ≥ 0.3 IU/kg/day glargine	Proportion of control subjects using ≥ 0.3 IU/kg/ day glargine	29, using any glargine	105, using any glargine		Mean daily dose: dividing total insulin units prescribed for duration of observation and weight	≥0.3 IU/kg/day glargine	5.46	0.45-66.1

ESM Table 4. Relative risk estimations for breast cancer among different duration and dose categories within insulin treatment groups (Continued)

Author year	Exposure	Comparator	Breast cancer (n) exposure	Breast cancer (n) comparator	Definition of duration	Definition of dose	Category	Risk estimate*	95 % CI
Detemir – no detemir: Hazard Ratio									
Fagot et al, 2013h [47]	Detemir users	Other int-/ long-acting insulin only users	NR per category, 38 total	NR		Similar to Fagot 2013 G	<14000 IU	1.13	0.66-1.96
							14000-27000 IU	1.02	0.51-2.03
							>27000 IU	1.04	0.48-2.26
Human insulin- no human insulin: Hazard Ratio									
Fagot et al, 2013i [47]	Intermediate-acting HI users	Other int-/ long-acting insulin only users	NR per category, 15 total	NR		Similar to Fagot 2013 G	<14000 IU	1.21	0.56-2.60
							14000-27000 IU	1.51	0.61-3.72
							>27000 IU	NE	NE
Ruiter et al, 2012d [64]	Non-glargine insulin users	Human insulin only users	3	NR		Stratified for median dose of first insulin prescription	<median dose	NE	NE
			29	NR			=median dose	0.90	0.70-1.15
			16	NR			>median dose	0.10	0.02-0.47

*Risk estimate are adjusted for covariates as stated in supplementary table 3. Covariates used in the various analyses are the same within one study. Studies are first ordered by type of exposure and then by type of risk estimate. Abbreviatios: NR= not reported, NE= not estimated, IU= international unit, NIAD= non-insulin anti-diabetic drug. For all exposure groups, the comparison group is no use of that insulin, except for insulin use versus NIAD use

ESM Table 5. Quality evaluation of the epidemiological studies included in the systematic review*

Author, Year	Bias			Risk of bias (4)	Power (5)
	Selection bias (1)	Information bias (2)	Confounding bias (3)		
Bodmer et al, 2010 [41]	Time window bias; not matched on potential exposure time	Misclassification bias: no latency period	Not adjusted for physical activity, important risk factors for BC	High	Too low
Cleveland et al, 2012 [45]	Controls are only frequency matched to cases. Different participation rate among cases (82%) and controls (63%)	Recall bias: interview, no data on duration of exposure, misclassification bias: no latency period	Not adjusted for DM duration	High	Too low
Grimaldi-Bensouda et al, 2013 [49]	Survival bias: BC cases who survived 1-2 years		Not adjusted for physical activity	Low	Borderline
Koro et al, 2007 [54]	Controls were not sampled time-dependently: controls did not have a BC record at any time during their follow-up	Misclassification bias: no latency period, no data on duration of exposure	Not adjusted for BMI, DM duration, other DM medication, physical activity, important risk factors for BC	High	Too low
Mannucci et al, 2010 [58]		Misclassification bias: insulin was not necessarily initiated at start of follow-up, misallocation of exposure time: follow-up for cases included unexposed time as time is counted from start follow-up, while for controls exposure time is counted from actual start of insulin exposure.	Not adjusted for DM duration, physical activity, important risk factors for BC	High	Too low
Carstensen et al, 2012 [43]			Not adjusted for BMI, DM duration, other DM medication, physical activity, important risk factors for BC	Moderate	Adequate
Chang et al, 2011 [44]		Bias due to informative censoring: due to exclusive users design, switchers are excluded	Not adjusted for BMI, DM duration, physical activity, important risk factors for BC	Moderate	Too low
Colhoun et al, 2009 [5]		Intention-to-treat approach, no data on duration of exposure	Not adjusted for BMI, DM duration, other DM medication, physical activity, important risk factors for BC	High	Too low
Currie et al, 2009 [6]		No data on duration of exposure	Not adjusted for other DM medications, physical activity, important risk factors for BC	Moderate	Too low
Fagot et al, 2013 [47]		Misclassification bias: not censored if discontinued or switched	Not adjusted for BMI, other DM medications, physical activity, important risk factors for BC	High	Too low
Ferrara et al, 2011 [48]		No data on duration of exposure before and during cohort	Not adjusted for BMI and physical activity, important risk factors for BC	Moderate	Adequate

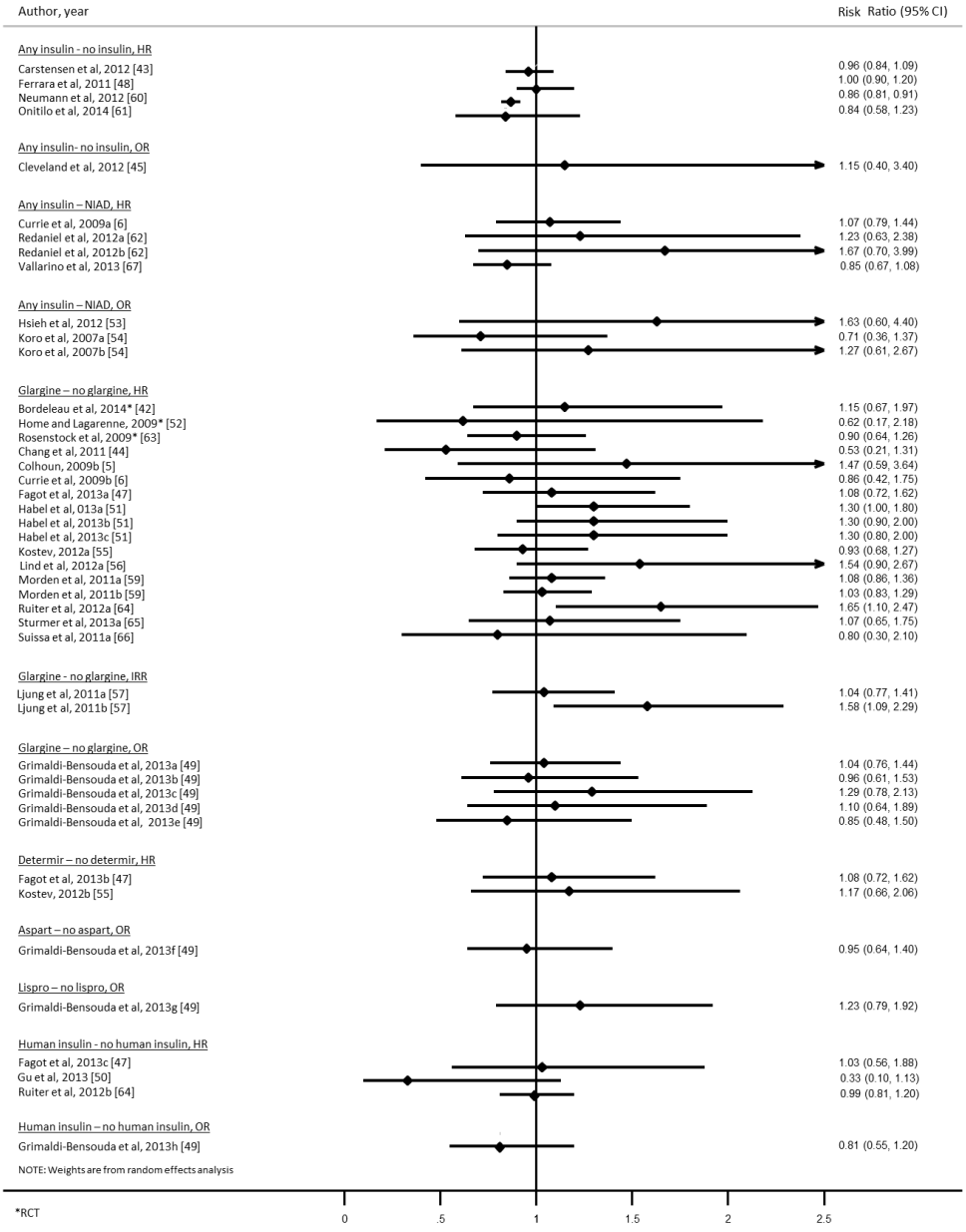
ESM Table 5. Quality evaluation of the epidemiological studies included in the systematic review* (Continued)

Author, Year	Bias			Risk of bias (4)	Power (5)
	Selection bias (1)	Information bias (2)	Confounding bias (3)		
Gu et al, 2013 [50]			Not adjusted for BMI, physical activity, important risk factors for BC	Moderate	Too low
Habel et al, 2013 [51]		Misclassification bias: no latency period	Not adjusted for BMI, DM duration, physical activity, important risk factors for BC	Moderate	Too low
Hsieh et al, 2012 [53]		No data on duration of exposure before and during cohort, bias due to informative censoring: due to exclusive users design, switchers are excluded	Not adjusted for BMI, DM duration, other DM medication, physical activity, important risk factors for BC	High	Too low
Kostev, 2012 [55] (Letter)		No information to identify potential risk of bias, misclassification bias: no latency period	Not adjusted for BMI, DM duration, other DM medication, physical activity, important risk factors for BC	High	Too low
Lind et al, 2012 [56]		Misclassification bias: no latency period	Not adjusted for DM duration, other DM medication, physical activity, important risk factors for BC	Moderate	Too low
Ljung et al, 2011 [57]		Intention-to-treat approach, no data on duration of exposure before and during cohort	Not adjusted for other DM medication, physical activity, important risk factors for BC	High	Low
Morden et al, 2011 [59]		Intention-to-treat approach, no data on duration of exposure before cohort	Not adjusted for DM duration, other DM medication, physical activity, important risk factors for BC	High	Low
Neumann et al, 2012 [60]		Immortal time bias, main study outcome: bladder cancer, no data on duration of exposure before and during cohort	Not adjusted for BMI, DM duration, physical activity, important risk factors for BC	High	Adequate
Onitilo et al, 2014 [61]		No data on duration of exposure, misclassification bias: no latency period, no proper exposure-comparison	Not adjusted for other DM medication, physical activity, important risk factors for BC	High	Too low
Redaniel et al, 2012 [62]			Not adjusted for other DM medication, physical activity, important risk factors for BC	Low	Too low
Ruiter et al, 2012 [64]			No adjustment for BMI, DM duration, physical activity, important risk factors for BC	Moderate	Too low
Sturmer et al, 2013 [65]			Not adjusted for BMI, DM duration, physical activity, important risk factors for BC	Moderate	Too low
Suissa et al, 2011 [66]		Intention-to-treat approach	Not adjusted for physical activity, important risk factors for BC	Moderate	Too low

Author, Year	Bias			Risk of bias (4)	Power (5)
	Selection bias (1)	Information bias (2)	Confounding bias (3)		
Vallarino et al, 2013 [67]		Bias due to informative censoring: due to exclusive users design, switchers are excluded	Not adjusted for age, BMI, DM duration, other DM medication, physical activity, important risk factors for BC	High	Low
Bordeleau et al, 2014 [42]		Not designed to study cancer outcome	No data on physical activity and important risk factors for BC. Other important covariates were similar at baseline among the treatment arms.	Low	Too low
Dejgaard et al, 2009 [46]		Misclassification bias: no latency period	No data on physical activity and important risk factors for BC. Other important covariates were similar at baseline among the treatment arms.	Low	Too low
Home and Lagarenne 2009 [52]		Misclassification bias: no latency period	NR	Moderate	Too low
Rosenstock et al, 2009 [63]		Not designed to study cancer outcome; misclassification bias: no latency period	NR; important covariates were similar at baseline among the treatment arms	Low	Too low

Abbreviations: NR= not reported, NE= not estimated, BC=breast cancer, DM= diabetes mellitus
Evaluation of loss to follow-up in cohort studies and selection of appropriate exposure and comparison groups in cohort studies and cases and controls in case-control studies. If cases were not matched to controls on calendar time and potential exposure time, we considered if time window bias could be present.
Evaluation of misclassification of exposure and outcome. It was determined whether exposure was measured cumulative over time, if investigators censored for switching or discontinuation of insulin treatment and whether a latency time was included.
Evaluation of adequate dealing with important risk factors in the analyses.
Risk of bias is summarized in low, moderate and high based on a (subjective) qualitative evaluation of selection, information and confounding bias.
Evaluation of whether the power of the study, given the number of exposed patients and number of BC events, was at least 80% to be able to detect a HR or OR of 1.2.
* More detailed information on the method of the quality evaluation can be found in ESM 3.

ESM Figure 1. Forest plot of breast cancer risk among insulin (analogues) users stratified by treatment group and type of effect estimate



Chapter 4

Diabetes and breast cancer subtypes

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Summary

Introduction: Women with diabetes have a worse survival after breast cancer diagnosis compared to women without diabetes. This may be due to a different etiological profile, leading to the development of more aggressive breast cancer subtypes. Our aim was to investigate whether insulin and non-insulin treated women with diabetes develop specific clinicopathological breast cancer subtypes compared to women without diabetes.

Methods: This cross-sectional study included randomly selected patients with invasive breast cancer diagnosed in 2000-2010. Stratified by age at breast cancer diagnosis (≤50 and >50 years), women with diabetes were 2:1 frequency-matched on year of birth and age at breast cancer diagnosis (both in 10-year categories) to women without diabetes, to select ~300 patients with tumor tissue available.
Tumor MicroArrays were stained by immunohistochemistry for estrogen and progesterone receptor (ER, PR), HER2, Ki67, CK5/6, CK14, and p63. A pathologist scored all stains and revised morphology and grade. Associations between diabetes/insulin treatment and clinicopathological subtypes were analyzed using multivariable logistic regression.

Results: Morphology and grade were not significantly different between women with diabetes (n=211) and women without diabetes (n=101), irrespective of menopausal status. Premenopausal women with diabetes tended to have more often PR-negative (OR=2.44(95%CI:1.07-5.55)), HER2-negative (OR=2.84(95%CI:1.11-7.22)), and basal-like (OR=3.14(95%CI:1.03-9.60) tumors than the women without diabetes, with non-significantly increased frequencies of ER-negative (OR=2.48(95%CI:0.95-6.45)) and triple negative (OR=2.60(95%CI:0.88-7.67) tumors. After adjustment for age and BMI, the associations remained similar in size but less significant. We observed no evidence for associations of clinicopathological subtypes with diabetes in postmenopausal women, nor with insulin treatment in general.

Conclusions: We found no compelling evidence that women with diabetes, treated with or without insulin, develop different breast cancer subtypes than women without diabetes. However, premenopausal women with diabetes tended to develop breast tumors that do not express hormonal receptors, which are typically associated with poor prognosis.

Introduction

Diabetes mellitus and breast cancer are chronic diseases with increasing incidence in many countries [1,2]. Recent estimates indicate that diabetes prevalence is 9.1% among women in Europe [1] and life-time risk for breast cancer is 9.7% [3]. Most patients with diabetes (~90%) have type 2 disease, characterized by reduced insulin secretion and insulin resistance with diagnosis in late adulthood, while patients with type 1 diabetes are insulin deficient [4].

Several studies have investigated whether diabetes and/or insulin (analogue) treatment increase breast cancer risk [5-10] or affect prognosis [11-18], because of their potential impact on tumor progression through e.g. the insulin-like growth receptor pathway [5,19]. Women with diabetes have a 15-20% increased risk of breast cancer compared to women without diabetes [6-9], but no impact of insulin analogue treatment has been shown [5]. Breast cancer in women with diabetes is often diagnosed at an advanced stage compared to women without diabetes [13,14,20-22]. Moreover, overall mortality after breast cancer diagnosis has been shown to be 50% higher in women with diabetes compared to women without diabetes [11-16], even after adjustment for tumor stage [13,14,16]. However, studies that investigated the association between breast cancer-specific mortality and diabetes show inconsistent results [11,23-27].

Diabetes itself might have a direct effect on breast cancer prognosis due to physiological effects of hyperglycemia, or hyperinsulinemia, which is a hallmark of insulin resistance commonly observed in patients with type 2 diabetes [28,29]. It has been shown that cancer-specific survival was decreased for women with abnormal glycemic status [25,27] and that fasting insulin levels are associated with worse outcome (distant recurrence and death), independent of Body Mass Index (BMI) [30]. However, diabetes itself and its complications may also increase risk of overall mortality [4] and shared cancer-promoting factors in patients with diabetes, such as obesity and a sedentary lifestyle, increases also the risk of death from competing causes (metabolic/cardiovascular diseases).

Another reason for the worse breast cancer survival may be that women with diabetes develop a more aggressive or less treatment-responsive tumor subtype. It has already been shown that hormone-related breast cancer and diabetes risk factors, such as obesity, are associated with the development of ER-negative breast cancer subtypes [31,32]. Insulin interacts with estrogens; there is experimental support that insulin may enhance estrogen production, stimulating the development of ER-positive breast cancer [19]. Furthermore, the promotion of tumor cell growth upon insulin exposure may differ by breast cancer subtype; we know from *in vitro* studies that mitogenic potential of insulins depends on the type of breast cancer cell line [5,33]. Although breast cancer subtypes have been extensively studied in the general population [31], few studies have assessed breast cancer subtypes in women with diabetes.

The aim of this study is to determine whether breast cancer patients with diabetes have a specific clinicopathological tumor subtype compared to those without diabetes, and whether the use of insulin is related to this.

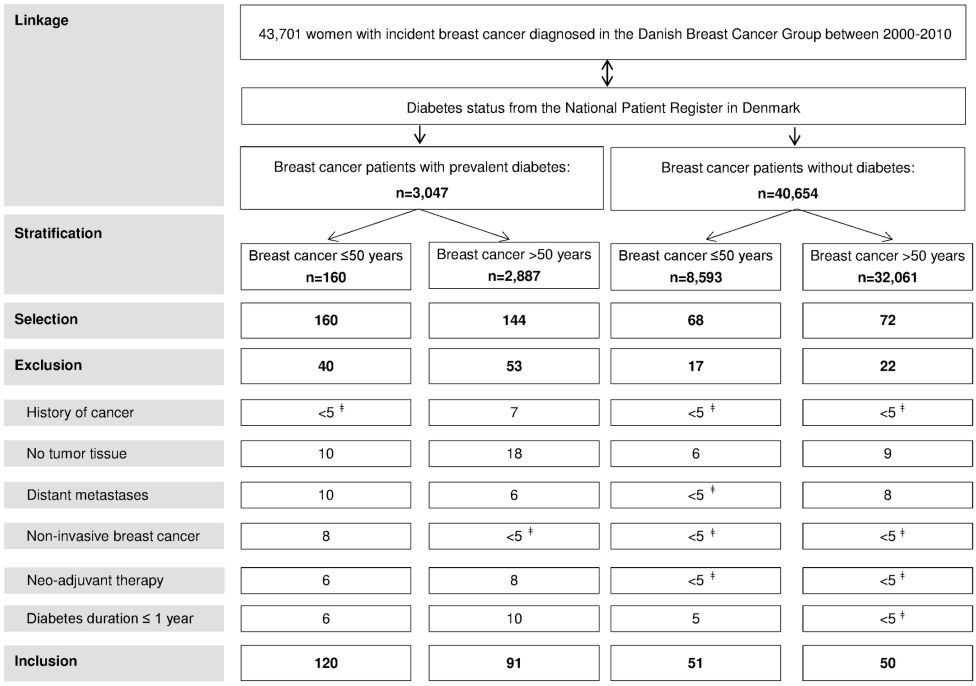
Methods

The study protocol was approved by the Science Ethics Committee of the Region Midtjylland in Denmark (M-20110198). The Science Ethics Committee of the Region Midtjylland in Denmark approved that informed consent for this study was not obtained; however, all women had the possibility to opt-out from research through the nation-wide registry. Tumor tissue of the women had been collected for diagnostic or therapeutic purposes around the time of breast cancer diagnosis. This tissue is stored in biobanks and may be used for research ('secondary use') as long as coded and anonymous to the researcher. No tissue was used against the will of the patients (women who opt-out with regard to tissue use for future scientific purposes were excluded (http://sundhedsdatastyrelsen.dk/da/registre-og-services/vaevsanvendelsesregisteret); no risk was posed to the women as the tissue had already been removed; and tumor tissue and data were anonymous for the researcher.

Study design and patient selection

The study population consists of Caucasian women with and without diabetes, diagnosed with primary breast cancer between 2000 and 2010. The breast cancer patients were selected from a previously established nation-wide hospital-based cohort, by the Danish Breast Cancer Cooperative Group (DBCG) [34]. This cohort was linked to the National Patient Register in Denmark to identify women with and without diabetes, covering the years since 1977. In total, 43,701 women were diagnosed with incident breast cancer in 2000-2010 in the DBCG, of whom 3,047 had diabetes (7.0%). We used a cross-sectional study design with a randomly selected target population of 300 breast cancer patients. The selected women included breast cancer patients with diabetes (exposed) and without diabetes (non-exposed) sampled as follows: a random sample of women with diabetes in strata of age ≤50 and >50 years (1:1) at breast cancer diagnosis (stratification by age to increase the number of young women) frequency matched with women without diabetes from the same database (1:2) by year of birth and age at diagnosis (both in 10-year categories) (Figure 1). Twice as many women with diabetes were included as women without diabetes to allow analyses by insulin treatment. Patients with a history of other cancers, non-invasive or metastasized breast cancer, those treated with neo-adjuvant therapy, patients with diabetes diagnosed ≤1 year prior to their breast cancer diagnosis, and patients with no or insufficient tumor tissue were excluded.

Figure 1. Flow chart of patient identification and selection



Stratified by age at breast cancer diagnosis (≤50 and >50 years), women with diabetes were 2:1 frequency-matched on year of birth and age at breast cancer diagnosis (both in 10-year categories) to women without diabetes, to select ~300 patients with tumor tissue available. ǂ Exact numbers <5 cannot be shown according to regulations of Statistics Denmark.

Data collection

Age, menopausal status, year of breast cancer diagnosis and information on tumor and tumor treatment were obtained from the DBCG databank and the pathology register of the women. Only age, year of breast cancer diagnosis, and diabetes status were available at the time of patient identification. Diabetes status, diabetes type (1 or 2), and age at diabetes diagnosis, as well as data on socioeconomic status were collected by linkage with the National Patient Register (which included all medical diagnoses from 1977 onwards) in Denmark. Data on medication use, available from 1995 onwards, was obtained by linkage with the Danish Register of Medicinal Products Statistics. All linkages were done using codes which render the data anonymous to the researchers who do not have direct access to these source databases. Women were defined as oral contraceptive or hormone replacement users if at least 2 prescriptions of the drug were prescribed cumulatively in the period up to one year prior to breast cancer diagnosis. Additional information on height, weight, Body Mass Index (BMI), smoking, alcohol use, and HbA_1C levels (measure of average glucose levels) prior to breast cancer diagnosis were retrieved from electronic patient files and anonymized before inclusion in the database for the researchers. Formalin-

fixed, paraffin-embedded tissue samples of the primary tumors were retrieved from different Departments of Pathology in Denmark, for central pathology review and immunohistochemical (IHC) analyses.

Tumor review and IHC analyses

All formalin-fixed, paraffin-embedded tumors blocks of the primary tumor of each patient were collected and whole slides were stained with Hematoxylin and Eosin. The most representative tumor block was selected for the analyses. Hematoxylin and Eosin slides were reviewed by a breast pathologist for morphology and grade (VJ). Grade was scored following the modified Bloom-Richardson system.

For the IHC analysis, tissue microarrays with 2 cores of 2 mm per tissue block were constructed. Tissue microarrays 3µ slices were placed on superfrost+ glass slides, and stained and scored for ER, PR, HER2, Ki67, CK5/6, CK14, and p63. HER2 2+ tumors were evaluated using SISH (Silver In Situ Hybridization). Scoring of the IHC staining was performed by a breast pathologist (VJ). A 10% cut-off was used to define a positive staining for all markers, except Ki67: low if ≤14% and high if >14% according to the St Gallen guidelines of 2013 [35], and HER2: negative if 0/1+ and positive if 2+(SISH confirmed)/3+. Tumors were defined as basal-like if at least one out of three basal markers (CK14, CK5/6, P63) were positive. We also classified the tumors using the St Gallen guidelines of 2013 using ER, PR, HER2, and Ki67 [35,36].

Diabetes treatment classification

Diabetes status was determined based on medical diagnosis from the National Patient Register. Diabetes duration was defined as time from age of diabetes diagnosis till age of breast cancer diagnosis. Women with diabetes were classified as insulin users if at least 2 prescriptions of insulin were prescribed cumulatively in the period up to one year prior to breast cancer diagnosis. Exposure time was defined as time from age of start of insulin till age of breast cancer diagnosis. For women treated with other non-insulin antidiabetic drugs, the same method was used. Women with diabetes treated with insulin only were considered patients with type 1 diabetes, if they had a recorded diagnosis of type 1 diabetes (n=21), or a medical code was missing but they were under age 30 years at diabetes diagnosis (n=4). All other women with diabetes were considered type 2.

Imputation

For women with unknown menopausal status (n=5), age over 52 years [37] was used as a proxy for postmenopausal status. Missing values for BMI (n=51 in women with diabetes, n=42 in women without diabetes) were imputed using Multivariate Imputations by Chained Equations [38] in R studio with a predictive mean matching regression model for each analyzed dataset, imputing variables with ascending number of missing values; number of imputations=10, number

of iterations=25; see (S1 Table). We assumed that data was missing at random and could be imputed because of correlations with other variables (S2 and S3 Table). Variables derived from the DBCG, i.e., age of breast cancer diagnosis, year of breast cancer diagnosis, menopausal status (for analyses in all women), breast cancer treatment; the electronic patient files, i.e., smoking, alcohol, height, weight, HbA_1C levels; the National Patient Registry, i.e., diabetes type, diabetes duration, cardiovascular disease, microvascular disease, income, education; the Danish Register of Medicinal Products Statistics, i.e., diabetes medication, hormone replacement treatment and oral contraception use; and data on breast cancer characteristics and clinicopathological subtypes. In the subsequent analyses, we only included the variables relevant for the prediction of clinicopathological subtype, i.e. age, menopausal status, smoking, alcohol, BMI, HbA_1C, diabetes duration, oral contraception use and hormone replacement treatment.

Statistical analyses

Patient and breast cancer characteristics at diagnosis were compared between breast cancer patients with and without diabetes using chi-square tests. Multivariable logistic regression models were used to estimate the association between diabetes status or insulin treatment with primary breast cancer clinicopathological subtypes. We constructed separate logistic regression models for each exposure (diabetes or insulin) to evaluate tumor subtype (various definitions) as model-specific outcomes. Multinomial logistic regression models were used for tumor subtypes which consisted of >2 categories. We tested for heterogeneity between insulin and non-insulin users in analysis restricted to diabetes patients only. In the analyses comparing women with and without diabetes, potential covariates were added in a one by one-stepwise manner; however, none of the covariates changed the beta-estimate for diabetes with >10% for any of the subtype classifications, except for BMI in the analysis of PR status and ER-/PR- in premenopausal women. Nonetheless, we are also showing adjusted models with breast cancer subtypes for age and BMI, because previous literature has shown associations between age, BMI and breast cancer subtypes [31]. Models for grade were adjusted for age only.

Modifications of the associations between diabetes status and breast cancer subtypes by menopausal status, BMI, and diabetes type were assessed using interactions terms. Although we found no statistically significant interactions between menopausal status and diabetes status (the lowest p-value was 0.07 in the analyses of PR), we show results for pre- and postmenopausal women separately based on previous evidence for different risk profiles [31]. To exclude potential bias by the inclusions of women with type 1 diabetes we performed a sensitivity analysis excluding women with type 1 diabetes. Moreover, explorative analyses were performed within women with type 1 and type 2 diabetes. SAS Enterprise guide 4.2 for Windows was used for statistical analyses.

Results

This cross-sectional study consisted of 211 women with diabetes and 101 women without diabetes, all diagnosed with breast cancer and with tumor tissue available (Figure 1). Breast cancer patients with diabetes had a similar distribution of menopausal status (as a result of the age-stratified selection), but were more often obese (BMI ≥30) (p <0.0001), compared to those without diabetes (Table 1). The majority of women with diabetes (88.2%) were diagnosed with type 2 diabetes and the mean diabetes duration was 8.9 years (S3 Table). Twenty-five percent (n=53) of the women with diabetes were treated with insulin; including 18 combined with non-insulin antidiabetic drugs. The non-insulin users were treated with non-insulin antidiabetic drugs (35%) or diabetes was controlled by diet and exercise only (40%) (S3 Table). The mean duration of insulin use was 8.4 years (S3 Table). Insulin users (47% type 1 diabetes women) were more often premenopausal compared to non-insulin users (p=0.04); and insulin users with premenopausal breast cancer had lower BMI compared to those not treated with insulin (p=0.0003) (S4 Table).

Association between diabetes and clinicopathological breast cancer subtypes

Breast cancer patients with diabetes had a similar distribution of morphology, tumor size, and number of positive lymph nodes compared to those without diabetes (Table 1); also if stratified for menopausal status (S5 Table).

Premenopausal breast cancer patients with diabetes had more often PR-negative (OR=2.44(95%CI:1.07-5.55), p=0.03), HER2-negative (OR=2.84(95%CI:1.11-7.21), p=0.03), and basal-like (OR=3.14(95%CI:1.03-9.60), p=0.05) tumors than those without diabetes, with non-statically significant increased frequencies of ER-negative (OR=2.48(95%CI:0.95-6.45)) and triple negative (OR=2.60(95%CI:0.88-7.67) tumors (Table 2 and S6 Table). After adjustment for age and BMI, the associations remained similar in size but less statistically significant. We found no statistically significant associations between diabetes status and grade or Ki67, nor using the more refined St. Gallen subtyping (Table 2 and S6 Table). We found no modification of breast cancer subtype by BMI or diabetes type. Sensitivity analyses, in which women with type 1 diabetes were excluded, resulted in hazard ratios of the same direction and similar size (S7 Table). We did not find an association between any of the clinicopathological breast cancer subtypes and diabetes in postmenopausal women (Table 2). In analyses including all women, we only found statistically significant more basal-like tumors in women with diabetes compared to those without (OR=2.39(95%CI:1.07-5.35), p=0.03).

Table 1. Characteristics of breast cancer patients with and without diabetes

	Women with breast cancer		
	Diabetes (n=211)	**No Diabetes** (n=101)	**P** [d]
Age, median (IQ range) [a, b]			
≤ 50 years	47.0 (43.0-50.0)	47.0 (43.0-50.0)	
> 50 years	67.0 (60.0-75.0)	67.0 (62.0-73.0)	
	% (n)	**% (n)**	
Year of breast cancer diagnoses [a]			
2000-2002	12.8 (27)	6.9 (7)	
2003-2004	15.6 (33)	16.8 (17)	
2005-2006	17.5 (37)	33.7 (34)	
2007-2008	27.5 (58)	18.8 (19)	
2009-2010	26.6 (56)	23.8 (24)	
Menopausal status [b]			0.57
Pre	52.1 (110)	48.5 (49)	
Post	47.9 (101)	51.5 (52)	
BMI in kg/m^2 [c]			
Premenopausal women			0.0002
<25 (normal)	30.3 (27)	46.7 (14)	
≥25 (overweight)	24.7 (22)	50.0 (15)	
≥30 (obese)	44.9 (40)	<5 (<5) [‡]	
Postmenopausal women			0.005
<25 (normal)	22.5 (16)	55.2 (16)	
≥25 (overweight)	38.0 (27)	31.0 (9)	
≥30 (obese)	39.4 (28)	<14 (<5) [‡]	
Morphology			0.54
Ductal	75.8 (160)	70.3 (71)	
Lobular	7.6 (16)	10.9 (11)	
Other	16.6 (35)	18.8 (19)	
Tumour size in mm			
≤ 20	57.8 (122)	57.4 (58)	0.54
21-50	36.5 (77)	39.6 (40)	
>50	5.7 (12)	<5 (<5) [‡]	
Number of positive lymph nodes			0.50
0	50.3 (102)	54.0 (54)	
1-3	32.5 (66)	26.0 (26)	
>3	17.2 (35)	20.0 (20)	
Grade			0.03
Grade 1	20.3 (41)	19.0 (19)	
Grade 2	35.6 (72)	51.0 (51)	
Grade 3	44.1 (89)	30.0 (30)	
ER			0.08
Positive	77.6 (163)	86.1 (87)	
Negative	22.4 (47)	13.9 (14)	
PR			0.17
Positive	64.4 (136)	72.3 (73)	
Negative	35.6 (75)	27.7 (28)	
HER2			0.07
Positive	10.5 (22)	17.8 (18)	
Negative	89.5 (187)	82.2 (83)	

[a] Matching variable, [b] at breast cancer diagnosis, [c] closest measure prior to breast cancer diagnosis, [d] Chi-square test. Missing values are not shown, therefore the sum of the categories does not add up to the total number of patients for BMI, positive lymph nodes, grade, ER and HER2. [‡] Exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark. *IQ=interquartile range*, *BMI=Body Mass Index*.

Table 2. Crude and adjusted odds ratios for breast cancer clinicopathological subtypes of women with diabetes compared to women without diabetes in subgroups of menopausal status using (multinomial) logistic regression

Premenopausal women with breast cancer				
	Independent variable of exposure			
	Diabetes vs. No Diabetes		**Diabetes vs. No Diabetes**	
Dependent variable	**crude OR (95% CI)**	**P**	**adjusted OR* (95% CI)**	**P**
Grade 2 (vs. grade 1)	0.56 (0.22-1.42)	0.22	0.56 (0.22-1.42)	0.22
Grade 3 (vs. grade 1)	1.02 (0.40-2.61)	0.97	1.08 (0.41-2.86)	0.88
ER- (vs. ER+)	2.48 (0.95-6.45)	0.06	2.32 (0.86-6.31)	0.10
PR- (vs. PR+)	**2.44 (1.07-5.55)**	**0.03**	2.18 (0.92-5.17)	0.07
HER2- (vs. HER2+)	**2.84 (1.11-7.22)**	**0.03**	**2.94 (1.08-8.02)**	**0.04**
High ki67 (vs. low ki67)	1.23 (0.62-2.42)	0.55	1.17 (0.53-2.58)	0.70
Basal-like [a] (vs. non-basal-like)	**3.14 (1.03-9.60)**	**0.05**	**3.11 (0.98-9.86)**	**0.05**
ER+/PR- (vs. ER+/PR+)	2.10 (0.55-7.96)	0.28	1.77 (0.43-7.18)	0.42
ER-/PR- (vs. ER+/PR+)	**2.67 (1.02-7.00)**	**0.05**	2.46 (0.90-6.75)	0.08
Luminal B-like, HER2- [c] (vs. luminal A-like [b])	1.15 (0.47-2.82)	0.76	1.05 (0.40-2.73)	0.92
HER2+ [d] (vs. luminal A-like)	0.46 (0.17-1.23)	0.12	0.41 (0.14-1.20)	0.10
Triple negative [e] (vs. luminal A-like)	2.60 (0.88-7.67)	0.08	2.21 (0.71-6.69)	0.17
Postmenopausal women with breast cancer				
	Independent variable of exposure			
	Diabetes vs. No Diabetes		**Diabetes vs. No Diabetes**	
Dependent variable	**crude OR (95% CI)**	**P**	**adjusted OR* (95% CI)**	**P**
Grade 2 (vs. grade 1)	0.80 (0.32-2.04)	0.65	0.80 (0.31-2.03)	0.64
Grade 3 (vs. grade 1)	1.97 (0.72-5.39)	0.19	1.97 (0.72-5.39)	0.19
ER- (vs. ER+)	1.27 (0.52-3.14)	0.60	1.33 (0.52-3.40)	0.55
PR- (vs. PR+)	0.96 (0.48-1.93)	0.92	1.06 (0.51-2.19)	0.88
HER2- (vs. HER2+)	1.15 (0.43-3.13)	0.78	1.20 (0.40-3.59)	0.75
High ki67 (vs. low ki67)	1.11 (0.56-2.22)	0.77	1.06 (0.52-2.18)	0.87
Basal-like [a] (vs. non-basal-like)	1.62 (0.50-5.29)	0.43	1.73 (0.51-5.91)	0.38
ER+/PR- (vs. ER+/PR+)	0.79 (0.33-1.87)	0.59	0.89 (0.36-2.19)	0.79
ER-/PR- (vs. ER+/PR+)	1.20 (0.48-3.04)	0.69	1.29 (0.49-3.39)	0.60
Luminal B-like, HER2- [c] (vs. luminal A-like [b])	0.65 (0.29-1.44)	0.29	0.58 (0.25-1.35)	0.21
HER2+ [d] (vs. luminal A-like)	0.79 (0.28-2.26)	0.66	0.88 (0.28-2.71)	0.82
Triple negative [e] (vs. luminal A-like)	1.29 (0.41-4.00)	0.66	1.30 (0.40-4.20)	0.67

Logistic regression for tumor subtypes with 2 categories and multinomial logistic regression for tumor subtype with >2 categories as the dependent variable. [a] Positive for ≥1 of the basal markers CK56, CK14, and P63, [b] ER+, PR+, HER2-, low Ki67, [c] ER+, PR-, HER2- with high Ki67, [d] ER+ or ER-, PR+ or PR-, HER2+, [e] ER-, PR-, HER2-. * Adjusted for age and BMI (continuous), except for grade, which is adjusted for age only. *OR=Odds Ratio, CI=Confidence Interval.*

Association between insulin treatment and clinicopathological breast cancer subtypes

Tumor morphology, tumor size and number of positive lymph nodes did not differ between women with diabetes treated with or without insulin (S4 Table); similar results were found in analyses stratified for menopausal status (data not shown).

We observed no statistically significant evidence for the development of poor prognosis tumors among insulin users (Table 3 and S8 Table). Premenopausal women with diabetes not using insulin were more likely to develop ER-negative (OR=3.06(95%CI:1.30-7.20), p=0.01) and PR-negative (OR=2.98(95%CI:1.11-8.00), p=0.03) compared to women without diabetes, while ORs for ER and PR-negative tumors in insulin users compared to women without diabetes were only slightly increased (Table 3 and S8 Table). We performed explorative analyses separately in type 1 and type 2 insulin-treated premenopausal women with diabetes trying to understand these differences between insulin and non-insulin users. The associations between diabetes and tumor subtypes among type 1 diabetes insulin users were more in line with the findings in the non-insulin users (e.g. poor prognosis tumors), while we observed a suggestion that type 2 diabetes insulin users had better prognosis tumors (S8 and S9 Table). However, overall, there was no evidence for a statistically significant heterogeneity between insulin and non-insulin users for any of the clinicopathological subtypes in the analyses restricted to breast cancer patients with diabetes (Table 3). In addition, adjustment for age and BMI did not materially change the effect estimates or their 95% confidence intervals (S8 and S10 Table). In postmenopausal women, we observed no association of insulin, with breast cancer subtypes (Table 3). We did not have enough power to include subtypes using the more refined St Gallen criteria in the analyses stratified by menopausal status. In analyses including all women, we found significantly more basal-like tumors (OR=2.5(95%CI:1.09-5.74), p=0.03) and ER-/PR-negative tumors (OR=1.99(95%CI:1.00-3.95), p=0.05) in non-insulin users compared to women without diabetes.

Table 3. Crude and adjusted odds ratios for breast cancer clinicopathological subtypes of women with diabetes treated with or without insulin compared to women without diabetes in subgroups of menopausal status using (multinomial) logistic regression

Premenopausal women with breast cancer					
	Independent variable of exposure				
	Insulin * vs. No Diabetes		**No Insulin † vs. No Diabetes**		**Diabetes only Insulin vs. No Insulin**
Dependent variable	**crude OR (95% CI)**	**P**	**crude OR (95% CI)**	**P**	**p**
Grade 2 (vs. grade 1)	0.55 (0.18-1.68)	0.29	0.57 (0.21-1.58)	0.28	0.93
Grade 3 (vs. grade 1)	0.53 (0.16-1.74)	0.30	1.34 (0.49-3.67)	0.57	0.09
ER- (vs. ER+)	1.54 (0.45-5.24)	0.49	**2.98 (1.11-8.00)**	**0.03**	0.20
PR- (vs. ER+)	1.37 (0.47-4.00)	0.57	**3.06 (1.30-7.20)**	**0.01**	0.08
HER2- (vs. ER+)	**8.97 (1.10-73.36)**	**0.04**	2.16 (0.82-5.67)	0.12	0.19
High ki67 (vs. low ki67)	0.80 (0.32-1.96)	0.62	1.48 (0.72-3.05)	0.29	0.15
Postmenopausal women with breast cancer					
	Independent variable of exposure				
	Insulin * vs. No Diabetes		**No Insulin † vs. No Diabetes**		**Diabetes only Insulin vs. No Insulin**
Dependent variable	**crude OR (95% CI)**	**P**	**crude OR (95% CI)**	**P**	**P**
Grade 2 (vs. grade 1)	0.60 (0.12-2.96)	0.53	0.85 (0.32-2.25)	0.75	0.66
Grade 3 (vs. grade 1)	2.05 (0.43-9.78)	0.37	1.95 (0.69-5.55)	0.21	0.95
ER- (vs. ER+)	1.47 (0.39-5.58)	0.57	1.23 (0.38-3.15)	0.66	0.78
PR- (vs. ER+)	1.01 (0.34-3.01)	0.98	0.95 (0.46-1.96)	0.89	0.90
HER2- (vs. ER+)	0.83 (0.19-3.60)	0.80	1.26 (0.44-3.63)	0.67	0.56
High ki67 (vs. low ki67)	0.80 (0.26-2.46)	0.70	1.19 (0.58-2.45)	0.63	0.46

Logistic regression for tumor subtypes with 2 categories and multinomial logistic regression for tumor subtype with >2 categories as the dependent variable. [a] Positive for ≥1 of the basal markers CK56, CK14, and P63, [b] ER+, PR+, HER2-, low Ki67, [c] ER+, PR-, HER2- with high Ki67, [d] ER+ or ER-, PR+ or PR-, HER2+, [e] ER-, PR-, HER2-. * Women with diabetes treated with insulin (analogues) regardless the use of concomitant non-insulin antidiabetic drugs, † women with diabetes treated only with diet and exercise and users of non-insulin antidiabetic drugs only. *OR=Odds Ratio, CI=Confidence Interval.*

Discussion

We found no compelling evidence that women with diabetes develop different clinicopathological subtypes compared to women without diabetes. However, premenopausal breast cancer patients with diabetes tend to develop breast tumors that do not express hormonal receptors and basal-like tumors, which are typically associated with poor prognosis. The majority of the women in our population had type 2 diabetes mellitus, so the results are most applicable for these patients. We also found no strong evidence that insulin treatment is associated with clinicopathological subtypes; though the poor-prognosis tumors were more often occurring in premenopausal women with diabetes *not* using insulin and in type 1 diabetes insulin users.

Only a few studies have investigated breast cancer characteristics among women with diabetes [20,22,39,40]. Two previous studies stratified the results for menopausal status and they also found that premenopausal women developed more often tumors that were hormone receptor negative [22,39], after multivariable adjustment [39]. Overall results were consistent with ours, showing more ER-negative, PR-negative and HER2-negative tumors in women with diabetes, with relative frequencies of 1.5 to 2.5, but most differences were not statistically significant, except for PR [20,22] and ER, even after adjustment for BMI [40]. A few studies that reported tumor markers (ER, PR, and some HER2 status) among women with diabetes [11-13,26,41] compared (breast cancer) mortality or disease-free survival among women with and without diabetes as their primary objective. Therefore, only crude estimates of associations between diabetes and tumor subtype were reported and not stratified for menopausal status. Women included in these studies were mainly postmenopausal and no significant associations were found between tumor markers and diabetes status.

Studies on the association between diabetes treatment and breast cancer subtype are even more scarce. No difference in tumor stage and tumor subtype among glargine versus non-glargine users was previously described [42,43]. Studies that compared metformin users to women with diabetes treated with sulphonylurea or insulin (non-metformin) showed no difference in ER status [20,44], but sulphonylurea or insulin users presented more PR-negative tumors (63.0% versus 26.7%, p=0.041) [44] and more HER-2 positive (29.5% versus 21%, p=0.002) [20] than in the metformin-treated subgroup.

Our study was based on the comprehensive biobanks (archival tumor tissue from a randomly selected group of women), and databases available in Denmark, and included medication history at least five years prior to breast cancer diagnosis from prescription records, resulting in a patient selection minimally affected by survival, selection or ascertainment bias. Due to oversampling of young breast cancer patients, we could examine the association between diabetes and clinicopathological subtypes in both pre- and post-menopausal women. An experienced breast pathologist reviewed all tumor samples and we had complete data on IHC markers (including basal markers). All IHC stainings were validated and performed in one center and scored by the same breast pathologist, to prevent inter-laboratory and inter-observer variability [45,46] and to assure quality of the data. Additionally, data on risk factors such as BMI were obtained and effect estimates were adjusted for potential confounders.

Our study was only sufficiently powered (around 80%; likelihood-ratio test with a two-sided p-value of 0.05) to detect large differences between breast cancer subtypes, e.g. 80% versus 60% ER-positive tumors, in women with and without diabetes and therefore, subtle differences may not have been detected. Furthermore, given the design of our study, in which odds ratios may represent on overestimation of the real risks, validation using prospective cohort analyses is recommended. Unfortunately, we had insufficient power for separate analyses of diabetes type 1 and different insulin analogues. We had also limited power to investigate the duration/dose of

insulin exposure and the effect on breast cancer subtype. However, the majority of insulin users had prescriptions of insulin over several years.

We had no information on whether breast cancer patients were mammography screen-detected or not. Breast cancer subtype of screen-detected tumors differs from tumors found outside of screening [47] and there may be a higher non-participation for screening among postmenopausal women with diabetes compared to women without diabetes [48]. However, Danish national screening programs started only in 2007 for women aged 50-69 [49]. All statistically significant differences in our study were found in premenopausal women <52 years, which were mostly not screened, because the use of opportunistic screening in Denmark is low [49].

BMI, HbA_1C and other risk factors such as alcohol and smoking were collected from the medical records of patients and were incomplete. However, since we had extensive data on variables associated with e.g. BMI, we were able to impute missing values using multiple imputations. Although the ratios for observed and imputed BMI were similar, BMI could still be misclassified for some patients. However, we think that misclassification of BMI is unlikely to influence our results, since BMI did not affect the association between diabetes and breast cancer subtype, except for PR status in premenopausal women. Nevertheless, we have to interpret both our positive and null results with caution.

There may be several reasons why we found a stronger and significant association between hormone receptor negative tumors and diabetes in premenopausal compared to postmenopausal women. Differences in levels of BMI-related and reproductive hormones, i.e., factors related to menopausal status, such as insulin, estrogen and adipokine, may play a role in tumor subtype formation. However, in contrast to what we have observed in postmenopausal women, a previous study showed increased estrogen levels in women with diabetes [19], which would imply that postmenopausal women would more often develop ER-positive tumors.

For the interpretation of the results, it is important to realize that diabetes and BMI are strongly associated. Women with diabetes are more likely to be obese, and premenopausal obese women tend to develop hormone receptor negative tumors [50]. Such an association between BMI and hormone receptor negative breast cancer has not been observed in postmenopausal women. Our results on the association between diabetes and breast cancer subtypes are in line with these findings, even after adjustment for BMI. The same has been reported by two other studies [39,40], which might indicate that diabetes itself contributes to higher rates of hormone receptor negative breast cancer in obese women. Our observation that poor prognosis tumors are unlikely to occur more often in premenopausal women using insulin, is in line with the earlier reports that insulin (analogues) do not increase the risk of breast cancer overall [10]. However, more research is needed for type 1 diabetes.

Diabetes medication depends on the type of diabetes, as well as the severity (insulin dependent, no endogenous insulin versus insulin resistant, high levels of endogenous insulin) and duration of diabetes. Not much is known about the mechanism, by which insulin treatment would possibly influence the receptor phenotype of breast cancer. It has been shown that insulin can induce ER and PR expression, which leads to increased binding capacity of ER in MCF-7 breast cancer cell line [51]. This may suggest that women with diabetes treated with insulin would develop more ER and PR-positive tumors, which we did not observe. Moreover, the interpretation and translation of in vivo and in vitro studies to the human setting is difficult [5].

In summary, our findings suggest that premenopausal women with diabetes tend to develop triple negative and basal tumors, which are typically associated with poor prognosis. Though our study had limited power, our results warrant further investigation and future studies should stratify their analyses by menopausal status.

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Supplementary material

S1 Table. Average Body Mass Index of breast cancer patients in subgroups of menopausal status, in the ten imputed datasets (% (n))

	Premenopausal women with breast cancer	
	Diabetes (n=110×10)	**No Diabetes** (n=49×10)
BMI in kg/m^2 [a]		
<25 (normal)	33.0 (363)	48.6 (238)
≥25 (overweight)	27.3 (300)	43.9 (215)
≥30 (obese)	39.7 (437)	7.6 (37)

	Postmenopausal women with breast cancer	
	Diabetes (N=101×10)	**No Diabetes** (N=52×10)
BMI in kg/m^2 [a]		
<25 (normal)	26.1 (264)	49.2 (256)
≥25 (overweight)	36.7 (371)	32.1 (167)
≥30 (obese)	37.1 (375)	18.7 (97)

[a] Closest measure prior to breast cancer diagnosis. Imputation was done separately for pre- and postmenopausal women. *BMI=Body Mass Index.*

4

S2 Table. Characteristics of breast cancer patients with and without diabetes used for imputation of Body Mass Index

	Women with breast cancer	
	Diabetes (n=211)	**No Diabetes** (n=101)
Height, mean ± SD	165.4 ± 6.2	165.4 ± 6.4
Weight, median (IQ range) [a]	78.0 (67.0-88.0)	68.0 (60.0-75.0)
	% (n)	**% (n)**
Smoking [a]		
Never	47.4 (100)	42.6 (43)
Former	14.7 (31)	18.8 (19)
Current	20.9 (44)	15.8 (16)
Missing	17.1 (36)	22.8 (23)
Alcohol [a]		
No	36.0 (76)	16.8 (17)
Moderate, <7 glasses/week	28.4 (60)	37.6 (38)
Heavy, >7 glasses/week	10.9 (23)	15.8 (16)
Missing	24.6 (52)	29.7 (30)
Income [b]		
Low (<200,000 Danish Krone)	63.5 (134)	45.5 (46)
Medium (200,000-399,999)	32.2 (68)	47.5 (48)
High (≥400,000)	3.8 (8)	6.9 (7)
Missing	0.5 (1)	-
Education [c]		
Primary	42.6 (90)	27.7 (28)
Secondary	34.1 (72)	38.6 (39)
Tertiary	17.1 (36)	30.7 (31)
Missing	6.2 (13)	3.0 (3)
Oral contraceptive use [d]		
Yes	34.6 (73)	34.7 (35)
No	65.4 (138)	65.4 (66)
Hormone replacement therapy [d]		
Yes	45.5 (96)	44.5 (45)
No	54.5 (115)	55.5 (56)
Cardiovascular disease		
Yes	19.4 (41)	6.9 (7)
No	80.6 (170)	93.1 (94)
Breast cancer treatment		
Surgery only	15.0 (29)	18.3 (17)
Surgery, chemotherapy + endocrine therapy	39.9 (77)	39.8 (37)
Surgery and endocrine therapy	26.4 (51)	28.0 (26)
Surgery and chemotherapy	18.7 (36)	14.0 (13)

[a] Closest measure prior to breast cancer diagnosis, [b] in year of breast cancer diagnosis, [c] highest attained, [d] at least 2 prescriptions of the drugs were prescribed cumulatively in the period up to one year prior to breast cancer diagnosis. *IQ=interquartile range, SD=standard deviation.*

S3 Table. Patient characteristics and medication use among women with type 1 and type 2 diabetes *

	Women with breast cancer and diabetes		
	Diabetes† (n=211)	**Type 1 Diabetes** (n=25)	**Type 2 Diabetes** (n=186)
Diabetes Type, % (n)			
Type 1	11.8 (25)		
Type 2	88.2 (186)		
Age diabetes diagnosis, median (IQ range)	46.0 (34.0-58.0)	23.0 (20.0-28.0)	47.5 (38.0-61.0)
premenopausal	36.0 (30.0-45.0)	22.0 (20.0-27.0)	39.0 (32.5-45.0)
postmenopausal	59.0 (52.0-69.0)	29.0 (20.0-47.0)	61.0 (54.0-69.0)
Diabetes duration in years, mean ± SD	8.9 ± 7.7	22.3 ± 7.3	7.1 ± 5.8
Menopausal status, % (n) [a]			
Pre	51.9 (110)	76.0 (19)	48.9 (91)
Post	48.1 (101)	24.0 (6)	51.1 (95)
BMI in kg/m^2, (%) n [b]			
<25 (normal)	20.4 (43)	40.0 (10)	17.7 (33)
≥25 (overweight)	23.2 (49)	24.0 (6)	23.1 (43)
≥ 30 (obese)	32.2 (68)	<5 (<5) ‡	36.0 (67)
Missing	24.2 (51)	32.0 (8)	23.1 (43)
Hemoglobin A1C in %, % (n) [c]			
Pre-diabetes and controlled; 5.7-7.9	14.2 (30)	24.0 (6)	12.9 (24)
Uncontrolled; ≥ 8.0	12.8 (27)	44.0 (11)	8.6 (16)
Missing	73.0 (154)	32.0 (8)	78.5 (146)
Diabetes treatment, % (n) [d]			
Diet and exercise	39.8 (84)	-	45.2 (84)
Non-insulin antidiabetic drugs only	35.1 (74)	-	39.8 (74)
Insulin only	16.6 (35)	100.0 (25)	5.4 (10)
Non-insulin antidiabetic drugs and insulin	8.5 (18)	-	9.7 (18)
Exposure time in years, mean ± SD [e]			
Any antidiabetic drugs	6.8 ± 4.1	10.1 ± 3.5	6.0 ± 3.8
Insulin	8.4 ± 4.2	10.1 ± 3.5	6.9 ± 4.2
Non-insulin antidiabetic drugs	5.5 ± 3.6	-	5.5 ± 3.6
Insulin type, % (n)			
Human insulin	23.4 (50)	100.0 (25)	13.4 (25)
Insulin analogues	11.7 (25)	44.0 (11)	7.5 (14)
Metformin	29.4 (63)	-	33.9 (63)

[a] At breast cancer diagnosis, [b] closest measure prior to breast cancer diagnosis, [c] measured in varying time periods before breast cancer diagnosis, [d] at least 2 prescriptions of an antidiabetic drug were prescribed cumulatively in the period up to one year prior to breast cancer diagnosis, [e] defined as time from age of start of the antidiabetic drug till age of breast cancer diagnosis. * Used for imputation, † all women with diabetes, ‡ exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark. *IQ=interquartile range, SD=standard deviation, BMI=Body Mass Index.*

S4 Table. Characteristics of breast cancer patients with diabetes treated with and without insulin

	Women with breast cancer and diabetes		
	Insulin * (n=53)	**No Insulin** † (n=158)	**P**
Age, median (IQ range) [a]	48.0 (44.0-51.0)	50.0 (47.0-68.0)	
≤ 50 years	47 (43.0-49.0)	48.0 (44.0-50.0)	
> 50 years	65.0 (58.0-69.0)	68.0 (61.0-75.0)	
Menopause % (n) [a]			
no	64.2 (34)	48.1 (76)	0.04
yes	35.9 (19)	51.9 (82)	
Diabetes type % (n)			
Type 1	47.2 (25)	-	<0.0001
Type 2	52.8 (28)	100 (158)	
BMI in kg/m^2, median (IQ range) [b]			
premenopausal	24.3 (22.3-28.9)	30.7 (25.9-34.3)	
postmenopausal	28.3 (23.2-35.0)	29.1 (25.2-31.2)	
Morphology % (n)			0.49
Ductal	73.6 (39)	76.6 (121)	
Lobular	11.3 (6)	6.3 (10)	
Others	15.1 (8)	17.1 (27)	
Tumor size in mm % (n)			
≤ 20	62.3 (33)	56.3 (89)	0.73
21-50	32.1 (17)	38.0 (60)	
>50	<6 (<5) ‡	5.7 (9)	
Number of positive lymph nodes % (n)			
0	47.1 (24)	51.3 (78)	0.86
1-3	35.3 (18)	31.6 (48)	
>3	17.7 (9)	17.1 (26)	

[a] At breast cancer diagnosis, [b] closest measure prior to breast cancer diagnosis. * Women with diabetes treated with insulin (analogues) regardless the use of concomitant non-insulin antidiabetic drugs, † women with diabetes treated only with diet and exercise and users of non-insulin antidiabetic drugs only, ‡ exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark. *IQ=interquartile range, SD=standard deviation.*

S5 Table. Tumor characteristics of breast cancer patients with and without diabetes in subgroups of menopausal status

	Premenopausal women with breast cancer		
	Diabetes (n=110)	**No Diabetes** (n=49)	**P** [a]
	% (n)	**% (n)**	
Morphology			0.25
Ductal	78.2 (86)	71.4 (35)	
Lobular	<5 (<5) [‡]	10.2 (5)	
Other	18.2 (20)	18.4 (9)	
Tumor size in mm			0.71
≤ 20	56.4 (62)	63.3 (31)	
21-50	39.1 (43)	32.7 (47)	
>50	4.6 (5)	<5 (<5) [‡]	
Number of positive lymph nodes			0.57
0	46.4 (51)	46.9 (23)	
1-3	37.3 (41)	30.6 (15)	
>3	16.4 (18)	22.5 (11)	
	Postmenopausal women with breast cancer		
	Diabetes (n=101)	**No Diabetes** (n=52)	**P** [a]
	% (n)	**% (n)**	
Morphology			0.79
Ductal	73.3 (74)	69.2 (36)	
Lobular	11.9 (12)	11.5 (6)	
Other	14.9 (15)	19.2 (10)	
Tumor size in mm			0.18
≤ 20	59.4 (60)	51.9 (27)	
21-50	33.7 (34)	46.2 (24)	
>50	6.9 (7)	<5 (<5) [‡]	
Number of positive lymph nodes			0.75
0	54.8 (51)	60.8 (31)	
1-3	26.9 (25)	21.6 (11)	
>3	18.3 (17)	17.7 (9)	

[a] Chi-square test. Missing values are not shown, therefore the sum of the categories does not add up to the total number of patients for positive lymph nodes. [‡] Exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark.

S6 table. Numbers and proportions of breast cancer clinicopathological subtypes of breast cancer patients with diabetes, type 2 diabetes and without diabetes in subgroups of menopausal status

	Premenopausal women with breast cancer			Postmenopausal women with breast cancer		
	Diabetes * (n=110)	**Type 2 Diabetes** † (n=91)	**No Diabetes** (n=49)	**Diabetes** * (n=101)	**Type 2 Diabetes** † (n=95)	**No Diabetes** (n=52)
Tumor subtype	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**
Grade 1	22.9 (25)	25.6 (23)	18.4 (9)	17.2 (16)	16.9 (15)	19.6 (10)
Grade 2	33.0 (36)	31.1 (28)	46.9 (23)	38.7 (36)	39.3 (35)	54.9 (28)
Grade 3	44.0 (48)	43.3 (39)	34.7 (17)	44.1 (41)	43.8 (39)	25.5 (13)
ER+	74.3 (81)	74.4 (67)	87.8 (43)	81.2 (82)	80.0 (76)	84.6 (44)
ER-	25.7 (28)	25.6 (23)	12.2 (6)	18.8 (19)	20.0 (19)	15.4 (8)
PR+	64.6 (71)	62.6 (57)	81.6 (40)	64.4 (65)	63.2 (60)	63.5 (33)
PR-	35.5 (39)	37.4 (34)	18.4 (9)	35.6 (36)	36.8 (35)	36.5 (19)
HER2+	9.3 (10)	10.0 (9)	22.5 (11)	11.9 (12)	11.6 (11)	13.5 (7)
HER2-	90.7 (98)	90.0 (81)	77.6 (38)	88.1 (89)	88.4 (84)	86.5 (45)
Low ki67	50.0 (54)	50.6 (45)	55.1 (27)	61.0 (61)	58.5 (55)	63.5 (33)
High ki67	50.0 (54)	49.4 (44)	44.9 (22)	39.0 (39)	41.5 (39)	36.5 (19)
Non-basal-like	78.2 (86)	78.0 (71)	91.8 (45)	88.1 (89)	88.4 (84)	92.3 (48)
Basal-like [a]	21.8 (24)	22.0 (20)	<9 (<5) ‡	11.9 (12)	11.6 (11)	<9 (<5) ‡
ER+/PR+	64.2 (70)	62.2 (56)	81.6 (40)	64.4 (65)	20.0 (19)	63.5 (33)
ER+/PR-	10.1 (11)	12.2 (11)	<7 (<5) ‡	16.8 (17)	16.8 (16)	21.2 (11)
ER-/PR-	25.7 (28)	25.6 (23)	12.2 (6)	18.8 (19)	63.2 (60)	15.4 (8)
Luminal A-like [b]	43.8 (46)	43.7 (38)	46.9 (23)	50.0 (50)	47.9 (45)	44.2 (23)
Luminal B-like, HER2- [c]	21.9 (23)	21.8 (19)	20.4 (10)	24.0 (24)	25.5 (24)	32.7 (17)
HER2+ [d]	9.5 (10)	10.3 (9)	22.5 (11)	12.0 (12)	11.7 (11)	13.5 (7)
Triple negative [e]	24.8 (26)	24.1 (21)	10.2 (5)	14.0 (14)	14.9 (14)	9.6 (5)

[a] Positive for ≥1 of the basal markers CK56, CK14, and P63, [b] ER+, PR+, HER2-, low Ki67, [c] ER+, PR-, HER2- with high Ki67, [d] ER+ or ER-, PR+ or PR-, HER2+, [e] ER-, PR-, HER2-. Missing values are not shown, therefore the sum of the categories does not add up to the total number of patients. * All women with diabetes, † women with type 2 diabetes only, ‡ exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark.

S7 Table. Crude and adjusted odds ratios for breast cancer clinicopathological subtypes of women with type 2 diabetes compared to women without diabetes in subgroups of menopausal status using (multinomial) logistic regression

Premenopausal women with breast cancer				
	Independent variable of exposure			
	Type 2 Diabetes vs. No Diabetes		**Type 2 Diabetes vs. No Diabetes**	
Dependent variable	**crude OR (95% CI)**	**P**	**adjusted OR * (95% CI)**	**P**
Grade 2 (vs. grade 1)	0.48 (0.19-1.23)	0.13	**0.34 (0.12-0.96)**	**0.04**
Grade 3 (vs. grade 1)	0.90 (0.34-2.34)	0.83	0.66 (0.21-2.00)	0.46
ER- (vs. ER+)	2.46 (0.93-6.53)	0.07	2.36 (0.82-6.83)	0.11
PR- (vs. PR+)	**2.65 (1.15-6.13)**	**0.02**	**2.50 (1.00-6.26)**	**0.05**
HER2- (vs. HER2+)	**2.61 (1.00-6.82)**	**0.05**	2.62 (0.90-7.62)	0.08
High ki67 (vs. low ki67)	1.20 (0.60-2.42)	0.61	1.17 (0.53-2.58)	0.70
Basal-like [a] (vs. non-basal-like)	**3.17 (1.02-9.87)**	**0.05**	3.12 (0.93-10.48)	0.07
ER+/PR- (vs. ER+/PR+)	2.62 (0.69-10.00)	0.16	2.49 (0.59-10.44)	0.21
ER-/PR- (vs. ER+/PR+)	**2.74 (1.02-7.34)**	**0.04**	2.67 (0.91-7.86)	0.08
Luminal B-like, HER2- [c] (vs. luminal A-like [b])	1.15 (0.46-2.90)	0.77	1.04 (0.38-2.86)	0.94
HER2+ [d] (vs. luminal A-like)	0.50 (0.18-1.38)	0.18	0.46 (0.15-1.43)	0.18
Triple negative [e] (vs. luminal A-like)	2.54 (0.83-7.67)	0.10	2.05 (0.61-6.85)	0.25

Postmenopausal women with breast cancer				
	Independent variable of exposure			
	Type 2 Diabetes vs. No Diabetes		**Type 2 Diabetes vs. No Diabetes**	
Dependent variable	**crude OR (95% CI)**	**P**	**adjusted OR * (95% CI)**	**P**
Grade 2 (vs. grade 1)	0.83 (0.33-2.14)	0.70	0.88 (0.33-2.35)	0.80
Grade 3 (vs. grade 1)	2.00 (0.72-5.53)	0.18	1.88 (0.65-5.39)	0.24
ER- (vs. ER+)	1.38 (0.56-3.40)	0.49	1.43 (0.56-3.67)	0.46
PR- (vs. PR+)	1.01 (0.50-2.04)	0.97	1.09 (0.52-2.28)	0.81
HER2- (vs. HER2+)	1.19 (0.43-3.28)	0.74	1.04 (0.35-3.06)	0.96
High ki67 (vs. low ki67)	1.23 (0.61-2.48)	0.56	1.18 (0.57-2.44)	0.66
Basal-like [a] (vs. non-basal-like)	1.57 (0.48-5.21)	0.46	1.70 (0.49-5.88)	0.40
ER+/PR- (vs. ER+/PR+)	0.80 (0.33-1.92)	0.62	0.88 (0.35-2.19)	0.78
ER-/PR- (vs. ER+/PR+)	1.31 (0.52-3.31)	0.57	1.39 (0.53-3.65)	0.51
Luminal B-like, HER2- [c] (vs. luminal A-like [b])	0.72 (0.33-1.60)	0.42	0.64 (0.27-1.50)	0.30
HER2+ [d] (vs. luminal A-like)	0.80 (0.46-4.46)	0.69	0.89 (0.28-2.80)	0.85
Triple negative [e] (vs. luminal A-like)	1.43 (0.46-4.46)	0.54	1.42 (0.44-4.61)	0.56

Logistic regression for tumor subtypes with 2 categories and multinomial logistic regression for tumor subtype with >2 categories as the dependent variable. Sensitivity analyses excluding women with type 1 diabetes. [a] Positive for ≥1 of the basal markers CK56, CK14, and P63, [b] ER+, PR+, HER2-, low Ki67, [c] ER+, PR-, HER2- with high Ki67, [d] ER+ or ER-, PR+ or PR-, HER2+, [e] ER-, PR-, HER2-. * Adjusted for age and BMI (continuous), except for grade which is adjusted for age only. *OR=Odds Ratio, CI=Confidence Interval.*

S8 Table. Numbers and proportions of breast cancer clinicopathological subtypes of breast cancer patients with diabetes (type 1 and type 2) treated with or without insulin in subgroups of menopausal status.

	Premenopausal women with breast cancer				Postmenopausal women with breast cancer	
	Diabetes with Insulin* (n=34)	**Type 1 Diabetes with Insulin** * (n=19)	**Type 2 Diabetes with insulin** * (n=15)	**Diabetes without Insulin** † (n=76)	**Diabetes with Insulin** * (n=19)	**Diabetes without Insulin** † (n=82)
Tumor subtype	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**
Grade 1	29.4 (10)	<11 (<5) ‡	53.3 (8)	20.0 (15)	<19 (<5) ‡	16.9 (13)
Grade 2	41.2 (14)	42.1 (8)	40.0 (6)	29.3 (22)	31.3 (5)	40.3 (31)
Grade 3	29.4 (10)	47.4 (9)	<7 (<5) ‡	50.7 (38)	50.0 (8)	42.9 (33)
ER+	82.4 (28)	73.7 (14)	93.3 (14)	70.7 (53)	79.0 (15)	81.7 (67)
ER-	17.7 (6)	26.3 (5)	<7 (<5) ‡	29.3 (22)	<22 (<5) ‡	18.3 (15)
PR+	76.5 (26)	73.7 (14)	80.0 (12)	59.2 (45)	63.2 (12)	64.6 (53)
PR-	23.5 (8)	26.3 (5)	<21 (<5) ‡	40.8 (31)	36.8 (7)	35.4 (29)
HER2+	<5 (<5) ‡	<6 (<5) ‡	-	11.8 (9)	13.5 (7)	11.0 (9)
HER2-	96.9 (31)	94.4 (17)	100 (14)	88.2 (67)	86.5 (45)	89.0 (73)
Low ki67	60.6 (20)	47.4 (9)	78.6 (11)	45.3 (34)	63.5 (33)	59.3 (48)
High ki67	39.4 (13)	52.6 (10)	<22 (<5) ‡	54.7 (41)	36.5 (19)	40.7 (33)

Missing values are not shown, therefore the sum of the categories does not add up to the total number of patients for grade, ER, HER2, ki67. * Women with diabetes treated with insulin (analogues) regardless the use of concomitant non-insulin antidiabetic drugs, † women with diabetes treated only with diet and exercise and users of non-insulin antidiabetic drugs only, ‡ exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark.

S9 Table. Crude odds ratios for breast cancer clinicopathological subtypes of premenopausal women with type 1 or type 2 diabetes treated with insulin compared to women without diabetes using (multinomial) logistic regression.

Premenopausal women with breast cancer					
	Independent variable of exposure				
	Type 1 Diabetes with Insulin * vs. No Diabetes		**Type 2 Diabetes with Insulin † vs. No Diabetes**		**Diabetes only Type 1 vs. Type 2 with Insulin**
Dependent variable	**crude OR (95% CI)**	**P**	**crude OR (95% CI)**	**P**	**P**
Grade 2 (vs. grade 1)	1.57 (0.28-8.83)	0.61	0.29 (0.08-1.09)	0.07	0.08
Grade 3 (vs. grade 1)	2.38 (0.42-13.47)	0.33	**0.07 (0.01-0.62)**	**0.02**	**0.01**
ER- (vs. ER+)	2.56 (0.68-9.69)	0.17	0.51 (0.06-4.63)	0.55	0.17
PR- (vs. PR+)	1.59 (0.45-5.55)	0.47	1.11 (0.26-4.77)	0.89	0.67
HER2- (vs. HER2+)	4.92 (0.59-41.22)	0.14	NE	NE	0.96
High ki67 (vs. low ki67)	1.36 (0.47-3.94)	0.57	0.34 (0.08-1.35)	0.12	0.08

Logistic regression for tumor subtypes with 2 categories and multinomial logistic regression for tumor subtype with >2 categories as the dependent variable. * Women with type 1 diabetes treated with insulin (analogues) regardless the use of concomitant non-insulin antidiabetic drugs, † women with type 2 diabetes treated with insulin (analogues) regardless the use of concomitant non-insulin antidiabetic drugs. *OR=Odds Ratio, CI=Confidence Interval, NE= Not Estimated.*

S10 Table. Adjusted odds ratios for breast cancer clinicopathological subtypes of women with diabetes treated with or without insulin compared to women without diabetes in subgroups of menopausal status using (multinomial) logistic regression.

Premenopausal women with breast cancer					
	Independent variable of exposure				
	Insulin * vs. No Diabetes		**No Insulin † vs. No Diabetes**		**Diabetes only Insulin vs. No Insulin**
Dependent variable	**adjusted OR (95% CI)**	**P**	**adjusted OR (95% CI)**	**P**	**P**
Grade 2 (vs. grade 1)	0.55 (0.18-1.68)	0.29	0.58 (0.21-1.59)	0.29	0.99
Grade 3 (vs. grade 1)	0.52 (0.15-1.77)	0.30	1.52 (0.53-4.37)	0.44	0.07
ER- (vs. ER+)	1.55 (0.45-5.38)	0.49	2.86 (0.97-8.41)	0.06	0.24
PR- (vs. ER+)	1.39 (0.47-4.10)	0.55	**2.70 (1.05-6.96)**	**0.04**	0.13
HER2- (vs. ER+)	**8.98 (1.09-74.19)**	**0.04**	2.11 (0.71-6.25)	0.18	0.20
High ki67 (vs. low ki67)	0.79 (0.32-1.99)	0.62	1.46 (0.63-3.39)	0.37	0.15

Postmenopausal women with breast cancer					
	Independent variable of exposure				
	Insulin * vs. No Diabetes		**No Insulin † vs. No Diabetes**		**Diabetes only Insulin vs. No Insulin**
Dependent variable	**adjusted OR (95% CI)**	**P**	**adjusted OR (95% CI)**	**P**	**P**
Grade 2 (vs. grade 1)	0.53 (0.10-2.72)	0.44	0.83 (0.31-2.19)	0.70	0.91
Grade 3 (vs. grade 1)	1.91 (0.39-9.41)	0.43	1.96 (0.69-5.58)	0.21	0.81
ER- (vs. ER+)	1.65 (0.40-6.73)	0.49	1.36 (0.50-3.66)	0.55	0.64
PR- (vs. ER+)	1.10 (0.34-3.53)	0.87	1.01 (0.47-2.17)	0.99	0.94
HER2- (vs. ER+)	1.54 (0.21-11.28)	0.67	1.13 (0.36-3.49)	0.84	0.86
High ki67 (vs. low ki67)	0.74 (0.22-2.42)	0.61	1.18 (0.55-2.51)	0.67	0.49

Logistic regression for tumor subtypes with 2 categories and multinomial logistic regression for tumor subtype with >2 categories as the dependent variable. ORs were adjusted for age and BMI (continuous), except for grade which is adjusted for age only. * Women with diabetes treated with insulin (analogues) regardless the use of concomitant non-insulin antidiabetic drugs, † women with diabetes treated only with diet and exercise and users of non-insulin antidiabetic drugs only. *OR=Odds Ratio, CI=Confidence Interval.*

Chapter 5

The association of diabetes mellitus and insulin treatment with expression of insulin-related proteins in breast tumours

Submitted for publication

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Summary

Background: The insulin receptor (INSR) and the insulin growth factor 1 receptor (IGF1R) play important roles in the aetiology of both diabetes mellitus and breast cancer. We aimed to evaluate the expression of hormone and insulin-related proteins within or related to the PI3K and MAPK pathway in breast tumours of women with or without diabetes mellitus, treated with or without insulin (analogues).

Methods: Immunohistochemistry was performed on tumour tissue of 312 women with invasive breast cancer, with or without pre-existing diabetes mellitus, diagnosed in 2000-2010, who were randomly selected from a Danish breast cancer cohort. Women with diabetes were 2:1 frequency matched by year of birth and age at breast cancer diagnosis to those without diabetes. Tumour Microarrays were successfully stained for p-ER, EGFR, p-ERK1/2, p-mTOR, and IGF1R, and scored by a breast pathologist. Associations of expression of these proteins with diabetes, insulin treatment (human insulin and insulin analogues) and other diabetes medication were evaluated by multivariable logistic regression adjusting for menopause and BMI; effect modification by menopausal status, BMI, and ER status was assessed using interactions terms.

Results: We found no significant differences in expression of any of the proteins in breast tumours of women with (n=211) and without diabetes (n=101). Among women with diabetes, insulin use (n=53) was significantly associated with higher tumour protein expression of IGF1R (OR=2.36; 95%CI:1.02-5.52; p=0.04) and p-mTOR (OR=2.35; 95%CI:1.13-4.88; p=0.02), especially among women treated with insulin analogues. Menopause seemed to modified the association between insulin and IGF1R expression (p=0.07); the difference in IGF1R expression was only observed in tumours of premenopausal women (OR=5.10; 95%CI:1.36-19.14; p=0.02). We found no associations between other types of diabetes medication, such as metformin, and protein expression of the five proteins evaluated.

Conclusions: In our study, breast tumours of women with pre-existing diabetes did not show an altered expression of selected PI3K/MAPK pathway-related proteins. However, we observed an association between insulin treatment and increased p-mTOR expression, and in premenopausal women with increased IGF1R expression of breast tumours. This observation, if confirmed, might be clinically relevant since the use of IGF1R and mTOR inhibitors are currently investigated in clinical trials.

Background

Approximately 10% of women diagnosed with breast cancer have pre-existing diabetes mellitus, which may affect their breast cancer progression, prognosis and treatment options [1-10]. Insulin (and the Insulin Growth Factor axes) appears to be an important factor linking diabetes and breast cancer [11-13]. In patients with diabetes, insulin metabolism is altered. Type 2 diabetes is characterized by insulin resistance, and in earlier stages by hyperinsulinemia (high levels of endogenous insulin). Women with type 2 diabetes are usually treated with non-insulin antidiabetic drugs and/or insulin (analogues), while patients with type 1 diabetes are insulin deficient and therefore rely on chronic treatment with insulin (analogues).

Due to the high homology between the two isoforms of the insulin receptor (INSR-A and INSR-B) and the insulin growth factor 1 receptor (IGF1R), insulin can bind to INSR-A, INSR-B and IGF1R [14]. Insulin analogues are structurally transformed from human insulin and this may result in increased binding affinity towards the IGF1R [15, 16]. Phosphorylation of INSR-B, caused by insulin binding, preferentially induces metabolic signals, while phosphorylation of INSR-A and IGF1R by insulin, predominantly leads to activation of two main intracellular signalling pathways involved in tumorigenesis: mitogen-activated protein kinase (MAPK-ERK) and phosphatidylinositol 3-kinase (PI3K-AKT) [17]. One of the downstream proteins important for control of cell growth is mammalian target of rapamycin (mTOR), which can be activated by the PI3K or MAPK pathway via respectively extracellular signal-regulated kinases (ERK) or protein kinase B (AKT) [14].

In vitro and in vivo studies have shown that endogenous and exogenous insulin can stimulate tumour promotion via INSR and IGF1R. In vitro, insulin analogue stimulation increases proliferation of breast cancer cells due to enhanced IGF1R (and INSR) signalling, while exposure to human insulin showed low mitogenic potential[18]. Chronic treatment with insulin-like compounds (IGF1, insulin AspB10) with strong binding affinity towards the IGF1R, decreased the tumour latency time and showed increased MAPK-ERK signalling in a mammary gland mouse model, while insulin glargine and human insulin treatment did not significantly decrease the time for tumour development compared to the vehicle-treated mice [19]. In vivo studies in humans and rats have suggested that the capacity for stimulation of the metabolic pathways via phosphorylation of INSR-B by insulin is lost in individuals with hyperinsulinemia due to insulin resistance, whereas insulin stimulation of the MAPK pathway is unaffected or even enhanced [20, 21]. There is also some evidence that IGF1R is more often expressed in breast tumours of patients with type 2 diabetes [22, 23].

Insulin can also stimulate tumour promotion via other receptors such as the oestrogen receptor (ER) pathway. Due to insulin-ER/progesterone receptor (PR) crosstalk the insulin receptor substrate 1 (IRS1) and subsequently the PI3K and MAPK signalling cascades can be upregulated resulting in enhanced proliferation [13]. Previous studies showed that IGF1R expression is higher in oestrogen-dependent cell lines [17].

Little is known about diabetes/insulin exposure and protein signalling in tumours in the human setting. On the basis of the limited amount of literature we hypothesized that tumours of patients with diabetes mellitus have higher expression of proteins in the insulin signalling pathway, especially among those treated with insulin and/or insulin analogues. Specifically, we aimed to evaluate the expression of (downstream activated) proteins within or related to the PI3K and MAPK pathways.

Methods

Study design, patient selection and data collection

We conducted a cross-sectional study with a target population of ~300 breast cancer patients, randomly selected from an existing nationwide hospital-based cohort set up by the Danish Breast Cancer Cooperative Group (DBCG), of women with primary breast cancer (N=43,701) diagnosed between 2000 and 2010 [24]. Details on patient selection and methods of data collection have been described previously [25]. In short, the selected women included breast cancer patients with pre-existing diabetes (exposed) and without diabetes (non-exposed) sampled as follows: a random sample of women with diabetes in strata of age ≤50 and >50 years (1:1 ratio) at breast cancer diagnoses was frequency matched to women without diabetes from the same database (1:2 ratio) by year of birth and age at diagnosis (both in 10-year categories) (see Figure 1 chapter 4). Twice as many women with diabetes were included as women without diabetes to allow analysis by insulin treatment. Patients with a history of other cancers, non-invasive or metastasized breast cancer, those treated with neo-adjuvant therapy, patients with diabetes diagnosed ≤1 year prior to their breast cancer diagnosis, and patients with no or insufficient tumour tissue were excluded. Age, menopausal status and year of breast cancer diagnosis were obtained from the DBCG database. Only age, year of breast cancer diagnosis and diabetes status were available at the time of patient identification. Diabetes status, diabetes type (1 or 2), and age at diabetes diagnosis were collected by linkage with the National Patient Register in Denmark. Data on diabetes medication, available from 1995, were obtained by linkage with the Danish Register of Medicinal Products Statistics. Additional information on height, weight and Body Mass Index (BMI) prior to breast cancer diagnosis were retrieved from electronic medical records. The study protocol was approved by the Science Ethics Committee of the Region Midtjylland in Denmark (M-20110198).

Diabetes treatment classification

Diabetes status was determined based on medical diagnosis from the Danish National Patient Register. Diabetes duration was defined as time from age of diabetes diagnosis till age of breast cancer diagnosis. Women with diabetes were assigned to a treatment group if at least 2 prescriptions of an antidiabetic drug were prescribed in the period up to one year prior to breast

cancer diagnosis. Exposure time was defined as time from age of start of the antidiabetic drug till age of breast cancer diagnosis. Women with diabetes treated with insulin only were considered patients with type 1 diabetes, if they had a recorded diagnosis of type 1 diabetes (n=21), or if a medical code was missing but they were under age 30 years at diabetes diagnosis (n=4). All other women with diabetes were considered type 2. We compared women with diabetes who had a history of treatment with: insulin (human insulin and/or insulin analogues) vs. never treated with insulin; insulin with non-insulin antidiabetic drugs vs. insulin only, the untreated women were excluded; insulin analogues vs. human insulin only; any antidiabetic medication vs. diet and exercise only; metformin vs. no metformin, in women who had a history of treatment with non-insulin antidiabetic drugs only.

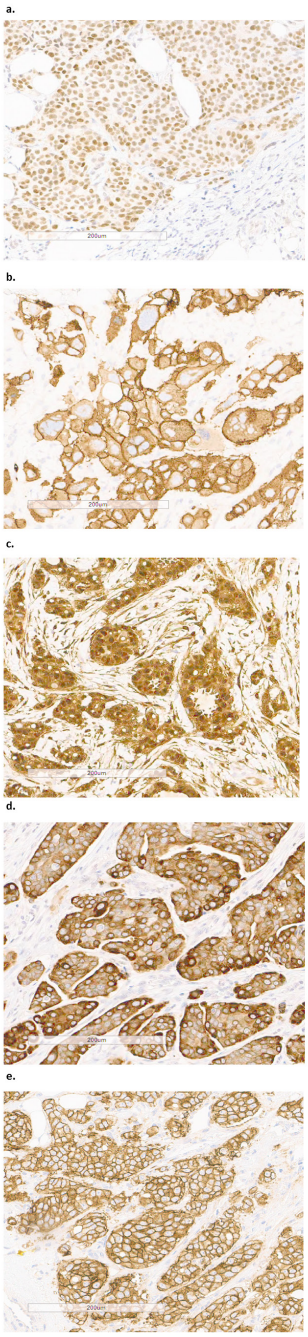
Tumour block collection and immunohistochemical (IHC) analyses

Formalin-fixed, paraffin embedded tissue samples of the primary tumours were retrieved from different pathology departments in Denmark. Morphology, grade, and clinical tumour subtype, immunohistochemically defined by oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status, were available from central pathology review [25]. All formalin-fixed, paraffin embedded tumour blocks of the primary tumour of each patient were collected, sectioned and hematoxylin and eosin (HE) stained. Two cores of 2mm were taken from the most representative tumour block of each patient for constructing duplicate Tissue Micro Arrays (TMAs), with one core of each patient on both TMAs. We chose hormone and insulin-related proteins within or related to pathways of interest (MAPK and PI3K) that were previously stained in the Netherlands Cancer Institute and/or reported in scientific articles with IHC application: p-ER, epidermal growth factor receptor (EGFR), INSR, IGF1R, p-ERK1/2, p-mTOR, phospho-ribosomal protein S6 kinase beta-1 (p-P70S6), and p-AKT. Antibodies for INSR, p-P70S6K, and p-AKT did not show sufficient validity and reliability on human breast tissue; staining was weak or showed variations in staining pattern. Varying dilutions of these antibodies and/or staining procedure (manual versus automated) did not lead to improvement. The antibodies for p-ER, EGFR, IGF1R, p-ERK1/2, and p-mTOR, were all developed and validated on human breast tissue by the Core Facility Molecular Pathology & Biobanking (CFMPB) of the Netherlands Cancer Institute. For each antibody a positive control was included.

Immunohistochemistry was performed on a BenchMark Ultra autostainer (Ventana Medical Systems). Briefly, 3 μm paraffin sections of TMAs were cut using a microtome, these sections were heated at 75°C for 28 minutes, and deparaffinised in the autostainer with 'EZ prep' solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for respectively 36 (p-mTOR), 64 (p-ERK1/2, EGFR, IGF1R) and 92 (p-ER) minutes at 95^{0}C. Primary antibody incubation times were 16 minutes (EGFR, IGF1R), 32 minutes (p-ER) and 1 hour (p-mTOR, p-ERK1/2). Details of the used antibodies, dilutions and localization of staining are summarized in Additional file: Table S1. Bound antibody

was detected using the UltraView Universal DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems).
Scoring of the IHC staining was performed by a breast pathologist (JS). The percentages of stained tumour cells were assessed for P-ER, EGFR, p-ERK1/2 and p-mTOR using a 10% step scale (0-100%). However, only the percentages of tumour cells stained with moderate to strong intensity were taken into account. We aimed to create a binary variable for a positive and negative staining. The cut-off for ER, PR and HER2 status is clear from daily practice (www.oncoline.nl) (<10% is negative)). However, for none of the other markers of interest there was a clinically defined cut-off available and we had to define cut-off values based on available literature, median expression levels (Table S2) and advice of an experienced breast pathologist (JW), before association analyses were carried out. P-mTOR was considered positive if cytoplasmic staining was present in ≥40% of the cells. For p-ER and EGFR was decided on a 10% cut-off for a positive nuclear and respectively membrane staining [26, 27]. P-ERK1/2 was considered positive if either nuclear or cytoplasmic staining was present in ≥10% of the cells [28]. IGF1R expression was scored negative for no staining or weak partial membrane or cytoplasmic staining and was scored positive if ≥10% of the tumour cells had a moderate or strong complete membrane or cytoplasmic staining [29, 30]. Figure 2 gives an overview of protein expression patterns of all proteins that were stained with moderate to strong staining. For all markers, discordant results between the two cores of each patient were revised and in case of a difference, the highest score was used for the analyses. If one core failed, the value of the remaining core was included in the analysis. Only the invasive part of the tumour, as judged by the pathologist, was considered when scoring the staining. When no (invasive) tumour cells were available, the result of the staining was coded as a missing value.

Figure 2. Patterns of immunohistochemical protein expression.
a. p-ER nuclear staining (70%), **b.** EGFR membrane staining (100%), **c.** p-ERK1/2 nuclear/cytoplasmic staining (100%), **d.** p-mTOR cytoplasmic staining (100%), **e.** IGFR strong complete membrane/cytoplasmic staining (≥10%)



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Statistical analyses

We hypothesized that diabetes, and in particular insulin use, would be associated with high(er) expression of IGF1R/EGFR and downstream activated proteins p-ERK1/2 and p-mTOR. Our primary analysis was therefore to test whether the expression of these proteins in breast tumours was dependent on diabetes status or insulin use, the latter analysis was restricted to women with diabetes only. We analysed these markers as a binary factor in a multivariable logistic regression model, using the cut-off value as specified above. For significant findings of continuously scored markers, the proportion of positively stained tumour cells were analysed as a continuous factor using a zero-inflated binomial (ZIB) model, as the data were not normally distributed. The ZIB model consists of a count component (negative binomial) and a binary component (logistic) and gives parameter estimates for both [31]. We did not perform this analysis for IGF1R since we did not continuously score the proportion of positively stained tumour for IGF1R.

Potential covariates, i.e. year of breast cancer diagnoses, age, menopausal status, BMI and diabetes duration, were individually added to the model and were only included if the beta-estimate for diabetes or insulin changed >10%. Menopause and BMI changed the beta for diabetes with >10% in the analyses of p-ER, EGFR, p-mTOR and IGF1R, and the beta for insulin in the analyses of p-ER, EGFR and p-ERK1/2. Therefore, for simplicity and consistency of between marker comparisons, all models were adjusted for menopause and BMI. For patients with unknown menopausal status (n=5), age over 52 years [32] was used as a proxy for postmenopausal status. Missing values for BMI (n=93) were imputed using Multivariate Imputations by Chained Equations (MICE) [33] in R studio. Methods of imputation have been described in more detail previously [25].

Modifications of the associations between diabetes status/insulin use and proteins of interest by menopausal status, BMI and ER status were assessed using interactions terms. To exclude potential bias by the inclusion of patients with type 1 diabetes we performed a sensitivity analysis comparing women with type 2 diabetes only to women without diabetes. We also tested for heterogeneity of expression of proteins between tumours of type 1 and type 2 diabetes patients using insulin. A p-value of <0.05 was defined as statistically significant. SAS Enterprise guide 4.2 for Windows was used for all analyses.

Results

The cross-sectional study consisted of 211 women with diabetes and 101 women without diabetes, all diagnosed with breast cancer and with tumour tissue available (see Figure 1 chapter 4). Patient and breast cancer characteristics at diagnosis have been published in detail previously and have been summarized in Table S3 and S4. Most women with diabetes were categorised as type 2 (88.2%). Immunohistochemistry could be evaluated in 93-96% of breast tumours, dependent on each marker (Table S2). In the evaluated tumours, positive protein expression was

found in 47% for p-ER, 9% for EGFR, 55% for p-ERK1/2, 59% for p-mTOR and 73% for IGF1R, respectively (Table S2).

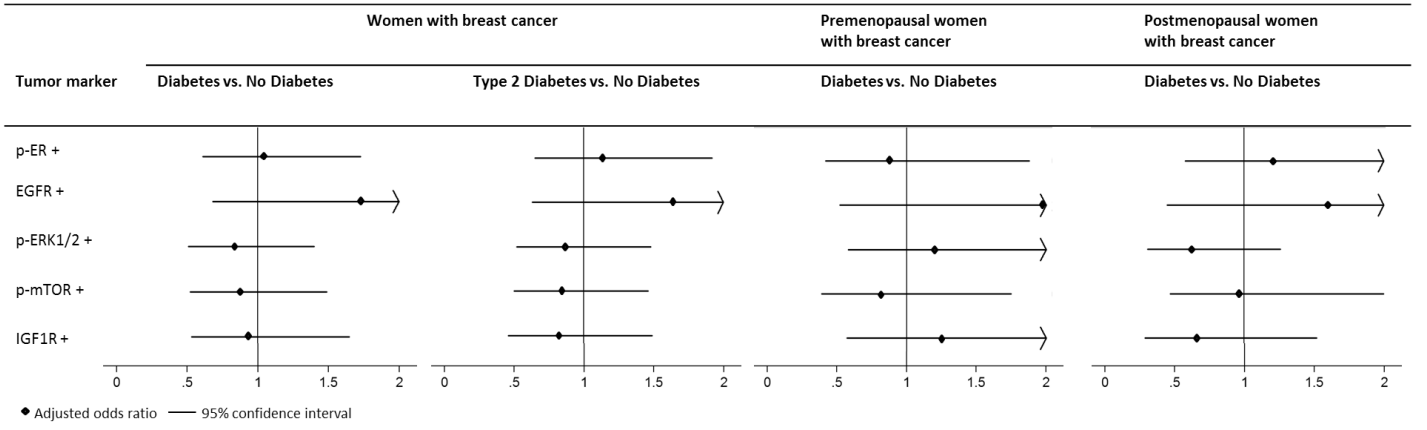
We found no significant differences in tumour expression of any of the selected proteins between women with and without diabetes (Figure 3, Table 1 and Table S5). Exclusion of women with type 1 diabetes gave similar results (Figure 3, Table S5 and S6). We found no effect modification of any of the proteins by menopause, ER status or BMI; except for p-ERK1/2, where there was some (non-significant) indication for interaction with menopause (p=0.17). After stratification for menopause (Figure 3, Table S5 and S6), p-ERK1/2 was not significantly associated with diabetes status, but we noticed that the direction of the effects of diabetes on p-ER, p-ERK1/2 and IGF1R differed in pre- and postmenopausal women.

Table 1. Crude and adjusted odds ratios for tumor protein expression status, by immunohistochemical markers, of women with diabetes compared to women without diabetes using logistic regression

Women with breast cancer				
	Independent variable of exposure			
	Diabetes vs. No Diabetes		**Diabetes vs. No Diabetes**	
Dependent variable *	**crude OR (95% CI)**	**P**	**adjusted OR [#] (95% CI)**	**P**
p-ER +	0.84 (0.51-1.37)	0.48	1.03 (0.61-1.73)	0.92
EGFR +	1.44 (0.59-3.52)	0.43	1.72 (0.68-4.33)	0.25
p-ERK 1/2 +	0.84 (0.52-1.37)	0.48	0.84 (0.51-1.40)	0.51
p-mTOR +	0.81 (0.49-1.33)	0.40	0.88 (0.52-1.49)	0.64
IGF1R +	0.90 (0.52-1.56)	0.70	0.94 (0.53-1.65)	0.82

* Logistic regression for tumor IHC marker as the dependent variable, with a negative staining of the tumor marker as reference category. [#] Adjusted for menopause (pre/post) at breast cancer diagnosis and BMI closest measure prior to breast cancer diagnosis (continuous). Women with diabetes were matched on age at breast cancer diagnosis to women without diabetes. *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth =factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor, OR=Odds Ratio, CI=Confidence Interval.*

Figure 3. Forest plots with crude odds ratios and 95% confidence intervals for tumor protein expression status, by immunohistochemical markers, of women with diabetes compared to women without diabetes using logistic regression



Twenty-five percent (n=53) of the women with diabetes were treated with insulin, of which 18 combined insulin with non-insulin antidiabetic drugs (Table S4). Among the insulin users, 28 were treated with human insulin only and 25 used insulin analogues with (n=22) or without human insulin (n=3). The non-insulin users (75%, n=158) were treated with non-insulin antidiabetic drugs (n=74) or diabetes was controlled by diet and exercise only (n=84). Any insulin use was significantly associated with higher expression of IGF1R (OR=2.36; 95%CI:1.02-5.52; p=0.04) and p-mTOR (OR=2.35; 95%CI:1.13-4.88; p=0.02; Figure 4, Table 2 and Table S7) in breast tumours. Additional analyses including the proportion of positively stained tumour cells as a continuous factor (using the ZIB model) gave similar results (data not shown); e.g. in the analyses for p-mTOR, the binary components explained most of the difference (estimate=-1.21, p=0.02), while the count component did not add much (estimate=0.03, p=0.80). Therefore, the logistic analyses were appropriate and using the data continuously did not improve the model. Expression of IGF1R significantly differed between insulin analogues users (n=28) and users of human insulin only (n=25) (Figure 4, Table S7 and S9). Insulin analogue users more often developed tumours that expressed IGF1R compared to human insulin only users (OR=4.94; 95%CI:1.11-21.92; p=0.04). The OR for p-mTOR was also higher among insulin analogue users, but not significantly different (OR=2.46; 95%CI:0.91-6.63; p=0.08) (Figure 4, Table S7 and S9).

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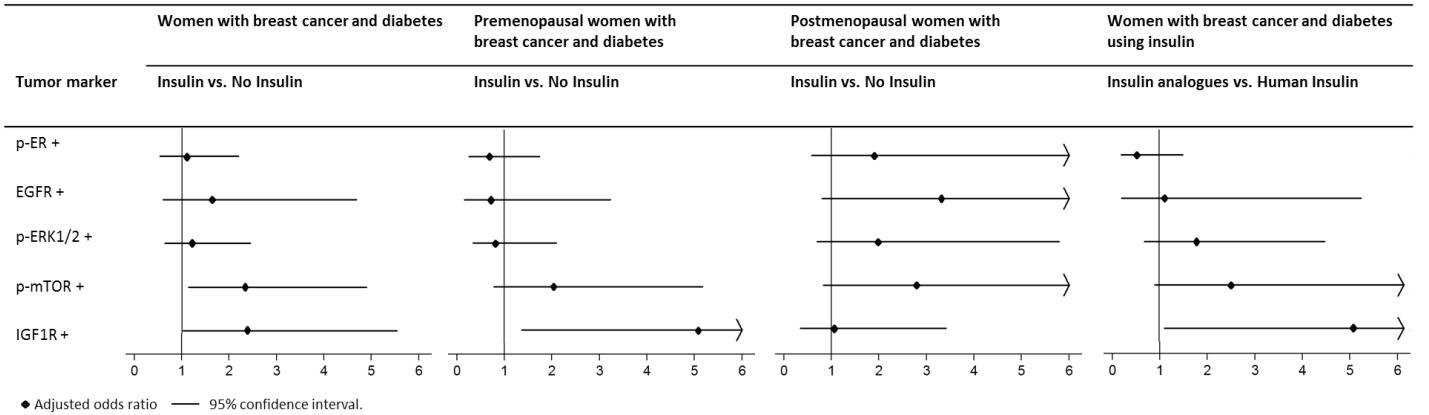
Table 2. Crude and adjusted odds ratios for tumor protein expression status, by immunohistochemical markers, of women with diabetes treated with insulin compared to women not treated with insulin using logistic regression

Women with breast cancer and diabetes				
	Independent variable of exposure			
	Insulin [§] vs. No Insulin [†]		**Insulin [§] vs. No Insulin [†]**	
Dependent variable *	**crude OR (95% CI)**	**P**	**adjusted OR [#] (95% CI)**	**P**
p-ER +	1.13 (0.38-2.19)	0.73	1.08 (0.53-2.19)	0.82
EGFR +	1.84 (0.69-4.91)	0.22	1.67 (0.60-4.67)	0.33
p-ERK 1/2 +	1.31 (0.68-2.53)	0.42	1.24 (0.63-2.44)	0.54
p-mTOR +	**2.41 (1.18-4.93)**	**0.02**	**2.35 (1.13-4.88)**	**0.02**
IGF1R +	**2.47 (1.07-5.67)**	**0.03**	**2.36 (1.02-5.52)**	**0.04**

* Logistic regression for tumor IHC marker as the dependent variable, with a negative staining of the tumor marker as reference category. [§] Women with diabetes treated with insulin (analogues) regardless the use of concomitant noninsulin antidiabetic drugs. [†] Women with diabetes treated only with diet and exercise and users of noninsulin antidiabetic drugs only. [#] Adjusted for menopause (pre/post) at breast cancer diagnosis and BMI closest measure prior to breast cancer diagnosis (continuous). *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth =factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor, OR=Odds Ratio, CI=Confidence Interval.*

Figure 4. Forest plots with crude odds ratios and 95% confidence intervals for tumor protein expression status, by immunohistochemical markers, of women with diabetes treated with insulin compared to women not treated with insulin using logistic regression



Menopause seemed to modify the association between insulin and IGF1R expression (p=0.07) and the difference in IGF1R expression between tumours of insulin and non-insulin users was only observed among premenopausal women with diabetes (OR=5.10; 95%CI:1.36-19.14; p=0.02; Figure 4, Table S8 and S9). We found no significant interaction between insulin use and ER status (p≥0.15) or BMI (p≥0.20). However, because the origin of the present breast cancer subtype classification is largely based on ER status; we confirmed that results were similar if analyses were stratified by ER-status (Table S8 and S9). Adjustment for ER status in the multivariable model did also not materially change the estimates, but adjustment for breast cancer subtype (Luminal A/ Luminal B/HER2-positive/triple negative) led to slightly stronger associations of insulin with IGF1R (OR=2.78; 95%CI:1.09-7.09; p=0.03) and p-mTOR (OR=3.42; 95%CI:1.43-8.17; p=0.006), with more expression of IGF1R and p-mTOR in triple negative, and less expression in HER2 positive tumours. We found no significant heterogeneity between tumour expression of the proteins of interest between diabetes type 1 and type 2 insulin users, except for p-ER (type 1 vs type 2: OR=0.28; 95%CI:0.08-0.95; p=0.04) (Table S7 and S9), but after adjustment for menopause and BMI this difference was non-significant.

We observed no statistically significant differences between expression of any of the proteins among tumours of women with diabetes treated with a combination of insulin and non-insulin-antidiabetic drugs compared to insulin-only users, nor did we find differences between tumours of women with diabetes treated with any diabetes medication compared to women with diabetes treated with diet and exercise only. In our study, 69% (n=51) of the women treated with non-insulin antidiabetic drugs only were treated with metformin (Table S4). We did not find a significant decreased effect of p-mTOR activation in tumours of metformin users (n=51) compared to non-insulin antidiabetic drug users not treated with metformin (n=23) (OR=0.57; 95%CI:0.21-1.56; p=0.27), nor did we find differences in any of the other proteins.

Discussion

We found no strong evidence that p-ER, EGFR, p-ERK1/2, p-mTOR, or IGF1R are differently expressed in breast tumours of women with and without diabetes. We showed that insulin treatment is associated with higher IGF1R and p-mTOR tumour expression in women with diabetes. Among insulin users, IGF1R was significantly more often expressed in tumours of women treated with insulin analogues compared to women treated with human insulin only. We found no strong evidence for an association between other types of diabetes medication, such as metformin, and any of the proteins that were assessed.

Insulin treatment was only associated with IGF1R expression in tumours of premenopausal women with diabetes. Previously, we found that premenopausal women with breast cancer and diabetes more often develop tumours that do not express hormonal receptors (especially among

women with type 1 diabetes)[25]. This might indicate that in women with tumours not expressing hormone receptors, the IGF1R signalling pathway might be an alternative way of breast cancer development, since this type of tumour is not dependent on the common ER/PR-signalling. We also found that ER is more often phosphorylated in women using insulin with type 2 diabetes compared to type 1 diabetes, which is in line with our previous findings that type 2 diabetes insulin users had more often ER-positive tumours compared to women with type 1 diabetes [25]. It has been suggested that phosphorylation of ER (at Ser^{118}) indicates that the ER signalling pathway in breast cancer is intact and that it is correlated with responsiveness of breast cancer to tamoxifen[27]. We did not find an association between metformin use and p-mTOR or any other of the examined proteins, while it has been suggested that metformin can decrease INSR and IGF1R signalling and can inhibit mTOR[34].

It should be noted that the specific proteins we investigated, especially IGF1R, are involved in signalling pathways that interfere with other growth factor receptor pathways such as ER, PR and EGFR. Therefore, expression of these proteins should be interpreted in the context of breast cancer subtype[30]. In our study, adjustment for ER status did not materially change the results and adjustment for breast cancer subtype led to slightly stronger associations of insulin with IGF1R and p-mTOR. We found no interaction between insulin use and ER status and we confirmed that results were similar when analysed by ER-status.

As far as we know, two previous studies in humans, with small sample size (n=39-40), examined protein or gene expression of the IGF1, IGF2, IGFBP3, INSR, IGF1R and downstream targets IRS1, IRS2 and mTOR in women with or without type 2 diabetes[22, 23]. Both studies found no association between these proteins and diabetes either, except for IGF1R protein expression which was found to be significantly higher in women with diabetes[23]**.** *In vitro* studies have shown that the PI3K signalling pathway[35-38] and the MAPK pathway[35, 36] are significantly upregulated after stimulation of insulin analogues compared to human insulin. In mammary gland tumours of mice, expression of IR, IGF1R and p-AKT was significantly higher in insulin or insulin analogues-treated compared to vehicle-treated mice, while expression of p-ERK was only increased among tumours of mice treated with insulin analogues[19]. Our results suggest that treatment with insulin and insulin analogues increases signalling via mTOR. Since we could not stain p-AKT and the PI3K and MAPK pathway interacts with many other proteins/pathways, we can only speculate about the actual signalling pathways involved. Since insulin analogues might have different binding affinity towards the IGF1R compared to (endogenous) human insulin[18, 39], this may explain the higher tumours expression of IGF1R in the insulin treated women, especially in women treated with insulin analogues. *In vivo* studies have demonstrated that tumour growth in mice with hyperinsulinemia, reflecting endogenous exposure, was mainly associated with PI3K/AKT/mTOR signalling[40]. However, we were unable to study endogenous insulin exposure since we did not have information on c-peptide levels, a measure of insulin secretion. Additionally, we cannot be certain that women in our reference group (without diabetes) have normal endogenous insulin and glucose levels since many women are living with undiagnosed diabetes[41].

Our study was based on the comprehensive biobanks (archival tumor tissue from a randomly selected group of women), and databases available in Denmark, and included medication history at least 5 years prior to breast cancer diagnosis from prescription records, resulting in a patient selection minimally affected by survival, selection or ascertainment bias. All stainings were validated and performed in one centre and scored by the same experienced breast pathologist, to prevent inter-laboratory and inter-observer variability [42, 43] and to assure quality and completeness of the data. We scored staining intensity and percentage positive tumour cells, and used the continuous expression data to validate our binary analyses. Median expression levels in our study corresponded with median expression levels and cut-offs used in previous studies examining these proteins [26-30]. The percentages of positive expression for p-ER, EGFR, p-ERK1/2, p-mTOR and IGF1R were also in line with previous published data, using similar population selection, IHC methods, and assessment criteria in primary invasive breast tumours [23, 27, 28, 30, 44]. Additionally, effect estimates were adjusted for potential confounders and analysed for potential effect modifiers and are therefore less likely to be distorted by the presence of other factors.

We had limited power to study differences of tumour protein expression among insulin users in women with type 1 and type 2 diabetes and between insulin analogues users and human insulin only users. We had also had no power to investigate the duration/dose of insulin exposure and the effect on tumour protein expression. The majority of insulin users had prescriptions of insulin over several years prior to breast cancer diagnosis (mean: 8.4 years), but we cannot guarantee the sequence of events (insulin exposure and subsequent tumour promotion) because of the potential lag time in the detection of the tumour. However, tumour size (an important factor for detection) was not related to diabetes status or insulin exposure, so it is unlikely that the associations we observed were due to reverse causation.

Due to the small frequencies of tumours that expressed EGFR, we could not interpret the results of this receptor. Frequencies of tumors that did not express the IGF1R are relatively small and 95%CIs are wide, therefore our findings might be due to chance. Unfortunately, antibodies targeting staining of the INSR and other proteins in the PI3K and MAPK pathway (such as AKT and p-P70S6K) did not work on our series of human breast tumour samples, as explained in the methods. Furthermore, we could not examine the phosphorylation state of the INSR compared to the IGF1R since there is only a non-specific p-INSR/p-IGF1R antibody available yet. At last, we considered that embedding and storage of tissue blocks may have been different between pathology laboratories, and this could have affected the results of the staining. However, this would only have confounded the analyses if diabetes status or insulin use would have been differentially distributed between laboratories or years of diagnosis, and this was not the case (Figure S1).

To conclude, we found that insulin treatment in women with diabetes is associated with p-mTOR tumour expression, and in premenopausal women with IGF1R tumour expression. However, more research is needed to confirm our findings and to explore the role of insulin signalling in breast cancer initiation and/or promotion in patients with diabetes, especially among those using insulin

or insulin analogues. This observation, if confirmed, might be clinically relevant since currently the use of IGF1R and mTOR inhibitors are investigated among breast cancer patients in clinical trials [17, 45, 46]. IGF1R and mTOR inhibitors might interfere with glucose metabolism in patients with diabetes and therefore monitoring for hyperglycaemia and dyslipidaemia may be important [47].

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Supplementary material

Table S1. Antibodies used for immunohistochemical assays

Protein name	Successful staining	Epitope	Clone	Manufacturer	Catalogue number	Dilution	Localization
p-ER	v	Ser118	16J4	Cell Signaling	2511	1/1200	Nuclear
EGFR	v		5B7	Roche	790-4347	Ready to use dispenser	Membrane
p-ERK1/2 (p-p44/42 MAPK)	v	Thr202/Tyr204	D13.14.4E	Cell Signaling	4370	1/400	Nuclear/Cytoplasmic
p-mTOR	v	Ser2448	49F9	Cell Signaling	2976	1/200	Cytoplasmic
p-P70S6K*		Thr389	1A5	Cell Signaling	9206	1/300	
p-Akt*		Thr308	C31E5E	Cell Signaling	2965	1/50	
p-Akt*		Ser473	D9E	Cell Signaling	4060	1/25	Cytoplasmic/Nuclear
IGF1R	v		G11	Roche	790-4346	Ready to use dispenser	Membrane/Cytoplasmic
INSR*			CT-3	Merck Millipore	05-1104	<1/50	Membrane

* antibody did not show sufficient validity and reliability on human breast tissue**.** P-Akt (Th 308) and p-P70-S6K (Thr389) showed variations in staining pattern, both antibodies appeared *not* to be validated for IHC-P by Cell Signaling. The p-Akt (Ser473) antibody was validated for IHC-P, but showed minimal positive signal on mamma tissue. The previously validated INSR antibody (MAB1139, Calbiochem) appeared not te be available anymore; therefore in data sheet recommended replacement was used. However, INSR staining with this replacement showed weak to no signal in tumor tissue although endothelial cells showed moderate to strong positivity. Using less diluted antibody and/or a manual staining procedure did not lead to increased signal. *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor, INSR=insulin receptor, p-AKT=phosphorylated protein kinase B, p-P70S6K=phosphorylated ribosomal protein S6 kinase.*

Table S2. Overview of the number of positively stained and unevaluable immunohistochemical markers; with for the evaluable cores the median percent of tumor cells with moderate to strong protein expression

Tumor IHC marker		n=312	%	Median (IQ range)
p-ER	Positive	136	47.1	0 (0, 40)
	Unevaluable	23	7.4	
EGFR	Positive	27	9.0	0 (0, 0)
	Unevaluable	13	4.2	
p-ERK1/2	Positive	163	54.7	10 (0, 40)
	Unevaluable	14	4.5	
p-mTOR	Positive	170	58.6	40 (0, 75)
	Unevaluable	22	7.1	
IGF1R *	Positive	214	72.8	
	Unevaluable	18	5.8	

* Median percent of tumor cells for IGF1R are not presented since we did not continuously score the percentages of tumors cells with moderate to strong staining. IHC for some of the tumors was not evaluable because the tumor tissue core did not include (invasive) breast tumor tissue or the core was missing. *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth =factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor.*

Table S3. Characteristics of breast cancer patients with and without diabetes and of insulin and non-insulin users

	Women with breast cancer						
	Diabetes (n=211)		**No Diabetes** (n=101)	**Insulin** [§] (n=53)		**No Insulin** [†] (n=158)
Age, median (IQ range) [a, b]						
≤ 50 years	47 (43-50)		47.0 (43-50)	47 (43-49)		48 (44-50)
> 50 years	67 (60-75)		67.0 (62-73)	65 (58-69)		68 (61-75)
BMI in kg/m², median (IQ range) [c]						
Premenopausal women	28.3 (23.9-33.5)	≠	25.2 (22.1-26.6)	24.3 (22.3-28.9)	≠	30.7 (25.9-34.3)
Postmenopausal women	29.0 (24.6-32.0)	≠	24.7 (21.0-27.0)	28.3 (23.2-35.0)		29.1 (25.2-31.2)
	% (n)		**% (n)**	**% (n)**		**% (n)**
Year of breast cancer diagnoses [a]						
2000-2002	12.8 (27)		6.9 (7)	20.7 (11)		10.1 (16)
2003-2004	15.6 (33)		16.8 (17)	13.2 (7)		16.5 (26)
2005-2006	17.5 (37)		33.7 (34)	17.0 (9)		17.7 (28)
2007-2008	27.5 (58)		18.8 (19)	28.3 (15)		27.2 (43)
2009-2010	26.6 (56)		23.8 (24)	20.8 (11)		28.5 (45)
Menopausal status [b]						
Pre	52.1 (110)		48.5 (49)	64.2 (34)	≠	48.1 (76)
Post	47.9 (101)		51.5 (52)	35.9 (19)	≠	51.9 (82)
Morphology						
Ductal	75.8 (160)		70.3 (71)	73.6 (39)		76.6 (121)
Lobular	7.6 (16)		10.9 (11)	11.3 (6)		6.3 (10)
Other	16.6 (35)		18.8 (19)	15.1 (8)		17.1 (27)
Grade						
Grade 1	20.3 (41)		19.0 (19)	26.0 (13)		18.4 (28)
Grade 2	35.6 (72)	≠	51.0 (51)	38.0 (19)		34.9 (53)
Grade 3	44.1 (89)		30.0 (30)	36.0 (18)		46.7 (71)
ER						
Positive	77.6 (163)		86.1 (87)	81.1 (43)		76.4 (120)
Negative	22.4 (47)		13.9 (14)	18.9 (10)		23.6 (37)
PR						
Positive	64.4 (136)		72.3 (73)	71.7 (38)		62.0 (98)
Negative	35.6 (75)		27.7 (28)	28.3 (15)		38.0 (60)
HER2						
Positive	10.5 (22)		17.8 (18)	<7 (<5) [‡]		11.4 (18)
Negative	89.5 (187)		82.2 (83)	92.2 (47)		88.6 (140)

[a] Matching variable, [b] At breast cancer diagnosis, [c] Closest measure prior to breast cancer diagnosis, [d] Chi-square test. Missing values are not shown, therefore the sum of the categories does not add up to the total number of patients for grade, ER and HER2. [§] Women with diabetes treated with insulin (analogues) regardless the use of concomitant noninsulin antidiabetic drugs. [†] Women with diabetes treated only with diet and exercise and users of noninsulin antidiabetic drugs only. [≠] statistically significant p <0.05.[‡] Exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark. *IQ=interquartile range, BMI=Body Mass Index, ER= Estrogen Receptor, PR=Progesterone Receptor, HER2=Human Epidermal growth factor Receptor 2.*

Table S4. Patient characteristics and medication use among women with type 1 and type 2 diabetes *

	Women with breast cancer and diabetes		
	Diabetes [§] (n=211)	**Type 1 Diabetes** (n=25)	**Type 2 Diabetes** (n=186)
Diabetes Type, % (n)			
Type 1	11.8 (25)		
Type 2	88.2 (186)		
Age diabetes diagnosis, median (IQ range)	46.0 (34.0-58.0)	23.0 (20.0-28.0)	47.5 (38.0-61.0)
premenopausal	36.0 (30.0-45.0)	22.0 (20.0-27.0)	39.0 (32.5-45.0)
postmenopausal	59.0 (52.0-69.0)	29.0 (20.0-47.0)	61.0 (54.0-69.0)
Diabetes duration in years, mean ± SD	8.9 ± 7.7	22.3 ± 7.3	7.1 ± 5.8
Menopausal status, % (n) [a]			
Pre	51.9 (110)	76.0 (19)	48.9 (91)
Post	48.1 (101)	24.0 (6)	51.1 (95)
BMI in kg/m^2, % (n) [b]			
<25 (normal)	20.4 (43)	40.0 (10)	17.7 (33)
≥25 (overweight)	23.2 (49)	24.0 (6)	23.1 (43)
≥ 30 (obese)	32.2 (68)	<5 (<5) [‡]	36.0 (67)
Missing	24.2 (51)	32.0 (8)	23.1 (43)
Diabetes treatment, % (n) [c]			
Diet and exercise	39.8 (84)	-	45.2 (84)
Non-insulin antidiabetic drugs only	35.1 (74)	-	39.8 (74)
Insulin only	16.6 (35)	100.0 (25)	5.4 (10)
Insulin and non-insulin antidiabetic drugs	8.5 (18)	-	9.7 (18)
Exposure time in years, mean ± SD [d]			
Any antidiabetic drugs	6.8 ± 4.1	10.1 ± 3.5	6.0 ± 3.8
Insulin	8.4 ± 4.2	10.1 ± 3.5	6.9 ± 4.2
Non-insulin antidiabetic drugs	5.5 ± 3.6	-	5.5 ± 3.6
Insulin type, % (n)			
Human insulin only	13.3 (28)	56.0 (14)	7.5 (14)
Insulin analogues only	1.4 (3)	-	1.6 (3)
Human insulin and insulin analogues	10.4 (22)	44.0 (11)	5.9 (11)
Metformin, % (n)			
Among non-insulin antidiabetic drug only users	24.2 (51)	-	27.4 (51)
Among insulin and non-insulin antidiabetic drug users	5.7 (12)	-	6.5 (12)

* Used for imputation. [a] At breast cancer diagnosis, [b] Closest measure prior to breast cancer diagnosis [c] at least 2 prescriptions of an antidiabetic drug were prescribed in the period up to one year prior to breast cancer diagnosis, [d] defined as time from age of start of the antidiabetic drug till age of breast cancer diagnosis. [§] All women with diabetes. [‡] exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark. *IQ=interquartile range, SD=standard deviation, BMI=Body Mass Index.*

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Table S5. Numbers and proportions of tumor protein expression status, by immunohistochemical markers, of women with diabetes, type 2 diabetes and without diabetes in all women and in subgroups of menopausal status

	Women with breast cancer			**Premenopausal women with breast cancer**		**Postmenopausal women with breast cancer**	
	Diabetes [§] (n=211)	**Type 2 Diabetes** [†] (n=186)	**No Diabetes** (n=101)	**Diabetes** (n=110)	**No Diabetes** (n=49)	**Diabetes** (n=101)	**No Diabetes** (n=52)
Tumor IHC marker	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**
p-ER -	54.4 (105)	52.6 (90)	50.0 (48)	63.1 (65)	54.4 (25)	44.4 (40)	46.0 (23)
P-ER +	45.6 (88)	47.4 (81)	50.0 (48)	36.9 (38)	45.7 (21)	55.6 (50)	54.0 (27)
EGFR -	90.0 (181)	90.4 (161)	92.9 (91)	90.3 (93)	93.8 (45)	89.8 (88)	92.0 (46)
EGFR +	10.0 (20)	9.6 (17)	7.1 (7)	9.7 (10)	<7 (<5) [‡]	10.2 (10)	<9 (<5) [‡]
p-ERK 1/2 -	46.7 (93)	46.6 (82)	42.4 (42)	41.8 (43)	44.9 (22)	52.1 (50)	40.0 (20)
p-ERK 1/2 +	53.3 (106)	53.4 (94)	57.6 (57)	58.3 (60)	55.1 (27)	47.9 (46)	60.0 (30)
p-mTOR -	43.1 (84)	44.5 (77)	37.9 (36)	43.7 (45)	35.6 (16)	42.4 (39)	40.0 (20)
p-mTOR +	56.9 (111)	55.5 (96)	62.1 (59)	56.3 (58)	64.4 (29)	57.6 (53)	60.0 (30)
IGF1R -	27.9 (55)	30.1 (52)	25.8 (25)	26.7 (28)	29.8 (14)	29.4 (27)	22.0 (11)
IGF1R +	72.1 (142)	69.9 (121)	74.2 (72)	73.3 (77)	70.2 (33)	70.7 (65)	78.0 (39)

The sum of the categories for the tumor markers does not add up to the total number of patients due to unevaluable stainings for some patients. [§] All women with diabetes, type 1 and type 2. † Women with type 2 diabetes only. [‡] Exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark. *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth =factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor.*

Table S6. Crude and logistic odds ratios for tumor protein expression status, by immunohistochemical markers, of women with type 2 diabetes compared to women without diabetes, and of women with and without diabetes in subgroups of menopausal status, using logistic regression

	Independent variable of exposure			
Dependent variable *	**crude OR (95% CI)**	**P**	**adjusted OR # (95% CI)**	**P**
Women with breast cancer				
	Type 2 Diabetes § vs. No Diabetes		**Type 2 Diabetes § vs. No Diabetes**	
p-ER +	0.90 (0.55-1.48)	0.68	1.12 (0.65-1.92)	0.69
EGFR +	1.37 (0.55-3.43)	0.50	1.64 (0.63-4.28)	0.31
p-ERK 1/2 +	0.85 (0.51-1.39)	0.51	0.88 (0.52-1.48)	0.62
p-mTOR +	0.76 (0.46-1.27)	0.29	0.85 (0.50-1.46)	0.56
IGF1R +	0.81 (0.46-1.41)	0.45	0.83 (0.46-1.49)	0.54
Premenopausal women with breast cancer				
	Diabetes vs. No Diabetes		**Diabetes vs. No Diabetes**	
p-ER +	0.70 (0.34-1.41)	0.31	0.88 (0.42-1.88)	0.75
EGFR +	1.61 (0.42-6.15)	0.48	2.03 (0.52-7.99)	0.31
p-ERK 1/2 +	1.14 (0.57-2.26)	0.71	1.19 (0.58-2.44)	0.63
p-mTOR +	0.71 (0.35-1.47)	0.36	0.82 (0.39-1.75)	0.61
IGF1R +	1.17 (0.55-2.50)	0.69	1.25 (0.57-2.77)	0.58
Postmenopausal women with breast cancer				
	Diabetes vs. No Diabetes		**Diabetes vs. No Diabetes**	
p-ER +	1.07 (0.53-2.13)	0.86	1.21 (0.58-2.51)	0.61
EGFR +	1.31 (0.39-4.40)	0.67	1.59 (0.45-5.63)	0.48
p-ERK 1/2 +	0.61 (0.31-1.23)	0.17	0.62 (0.31-1.26)	0.19
p-mTOR +	0.91 (0.45-1.83)	0.78	0.98 (0.47-2.00)	0.95
IGF1R +	0.69 (0.30-1.52)	0.35	0.67 (0.29-1.52)	0.34

* Logistic regression for tumor IHC marker as the dependent variable, with a negative staining of the tumor marker as reference category. § Women with type 2 diabetes only. # Adjusted for menopause (pre/post) at breast cancer diagnosis and BMI closest measure prior to breast cancer diagnosis (continuous). Women with diabetes were matched on age at breast cancer diagnosis to women without diabetes. *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth =factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor, OR=Odds Ratio, CI=Confidence Interval.*

Table S7. Number and proportion of tumor protein expression status, by immunohistochemical markers, of women with type 1 and type 2 diabetes treated with insulin (human and analogues) and without insulin

	Women with breast cancer and diabetes					
	Diabetes with Insulin* (n=53)	**Diabetes with insulin analogues** [#] (n=25)	**Diabetes with human insulin**[§] (n=28)	**Type 1 Diabetes with Insulin** * (n=25)	**Type 2 Diabetes with insulin** * (n=28)	**Diabetes without insulin**[†] (n=158)
Tumor IHC marker	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**
p-ER -	52.2 (24)	68.2 (15)	37.5 (9)	68.2 (15)	37.5 (9)	55.1 (81)
P-ER +	47.8 (22)	31.8 (7)	62.5 (15)	31.8 (7)	62.5 (15)	44.9 (66)
EGFR -	85.4 (41)	90.9 (20)	80.8 (21)	87.0 (20)	84.0 (21)	91.5 (140)
EGFR +	14.6 (7)	<10 (<5) [‡]	19.2 (5)	<14 (<5) [‡]	<17 (<5) [‡]	8.5 (13)
p-ERK 1/2 -	41.7 (20)	34.8 (8)	48.0 (12)	47.8 (11)	36.0 (9)	48.3 (73)
p-ERK 1/2 +	58.3 (28)	65.2 (15)	52.0 (13)	52.2 (12)	64.0 (16)	52.7 (78)
p-mTOR -	27.7 (13)	27.3 (6)	28.0 (7)	31.8 (7)	24.0 (6)	48.0 (71)
p-mTOR +	72.3 (34)	72.7 (16)	72.0 (18)	68.2 (15)	76.0 (19)	52.0 (77)
IGF1R -	16.0 (8)	<9 (<5) [‡]	22.2 (6)	<13 (<5) [‡]	19.2 (5)	32.0 (47)
IGF1R +	84.0 (42)	91.3 (21)	77.8 (21)	87.5 (21)	80.8 (21)	68.0 (100)

The sum of the categories for the tumor markers does not add up to the total number of patients due to unevaluable stainings for some patients. * Women with diabetes treated with insulin (analogues) regardless the use of concomitant non-insulin antidiabetic drugs. [#] Women with diabetes treated with insulin analogues regardless the use of concomitant human insulin (n=22) or noninsulin antidiabetic drugs. [§] Women with diabetes treated with human insulin only regardless the use of concomitant noninsulin antidiabetic drugs. [†] Women with diabetes treated only with diet and exercise and users of non-insulin antidiabetic drugs only. [‡] Exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark. *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth =factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor*

Table S8. Number and proportion of tumor protein expression status, by immunohistochemical markers, of women with diabetes treated with insulin and without insulin in subgroups of menopausal status and ER tumor status

	Women with breast cancer and diabetes							
	Premenopausal		Postmenopausal		ER positive		ER negative	
	Diabetes with Insulin* (n=34)	Diabetes without insulin† (n=76)	Diabetes With Insulin* (n=19)	Diabetes without insulin† (n=82)	Diabetes with Insulin* (n=43)	Diabetes without insulin† (n=121)	Diabetes with Insulin* (n=10)	Diabetes without Insulin† (n=38)
Tumor IHC marker	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**
p-ER -	63.3 (19)	63.0 (46)	31.3 (5)	47.3 (35)	43.2 (16)	47.8 (53)	88.9 (8)	78.4 (29)
P-ER +	36.7 (11)	37.0 (27)	68.8 (11)	52.7 (39)	56.8 (21)	52.3 (58)	<12 (<5) ‡	21.6 (8)
EGFR -	90.0 (27)	90.4 (66)	77.8 (14)	92.5 (74)	92.1 (35)	99.1 (115)	60.0 (6)	68.4 (26)
EGFR +	<11 (<5) ‡	9.6 (7)	<23 (<5) ‡	7.5 (6)	<8 (<5) ‡	<5 (<5) ‡	<41 (<5) ‡	31.6 (12)
p-ERK 1/2 -	43.3 (13)	41.1 (30)	38.9 (7)	55.1 (43)	42.1 (16)	45.6 (52)	<41 (<5) ‡	57.9 (22)
p-ERK 1/2 +	56.7 (17)	58.9 (43)	61.1 (11)	44.9 (35)	57.9 (22)	54.4 (62)	60.0 (6)	42.1 (16)
p-mTOR -	30.0 (9)	49.3 (36)	<24 (<5) ‡	46.7 (35)	18.9 (7)	36.6 (41)	60.0 (6)	81.1 (30)
p-mTOR +	70.0 (21)	50.7 (37)	76.5 (13)	53.3 (40)	81.1 (30)	63.4 (71)	<41 (<5) ‡	18.9 (7)
IGF1R -	<10 (<5) ‡	34.3 (25)	27.8 (5)	29.7 (22)	<11 (<5) ‡	18.8 (21)	<41(<5) ‡	72.2 (26)
IGF1R +	90.6 (29)	65.8 (48)	72.2 (13)	70.3 (74)	90.0 (36)	81.3 (91)	60.0 (6)	27.8 (10)

The sum of the categories for the tumor markers does not add up to the total number of patients due to unevaluable stainings for some patients. * Women with diabetes treated with insulin (analogues) regardless the use of concomitant non-insulin antidiabetic drugs. † Women with diabetes treated only with diet and exercise and users of non-insulin antidiabetic drugs only. ‡ Exact numbers <5 with percentages cannot be shown according to regulations of Statistics Denmark. *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth =factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor*

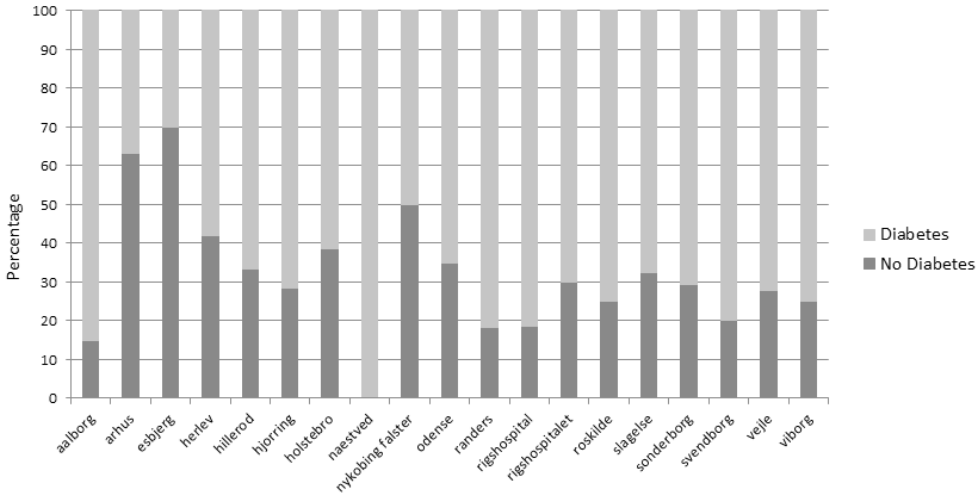
Table S9. Crude and adjusted odds ratios for tumor protein expression status, by immunohistochemical markers, of women with diabetes; treated with insulin analogues compared to women treated with human insulin; type 1 compared to type 2 insulin users; treated with insulin compared to women not treated with insulin in subgroups of menopausal status and in subgroups of ER tumor status using logistic regression

	Independent variable of exposure			
Dependent variable *	**crude OR (95% CI)**	**P**	**adjusted OR [#] (95% CI)**	**P**
Women with breast cancer and diabetes using insulin				
	Insulin analogue [‡] vs. human Insulin [¥]		**Insulin analogue [‡] vs. human Insulin [¥]**	
p-ER +	**0.28 (0.08-0.95)**	**0.04**	0.57 (0.22-1.49)	0.25
EGFR +	0.42 (0.07-2.42)	0.33	1.08 (0.23-5.13)	0.93
p-ERK 1/2 +	1.73 (0.54-5.54)	0.36	1.76 (0.70-4.38)	0.23
p-mTOR +	1.04 (0.29-3.74)	0.96	2.46 (0.91-6.63)	0.08
IGF1R +	3.00 (0.54-16.60)	0.21	**4.94 (1.11-21.92)**	**0.04**
Women with breast cancer and diabetes using insulin				
	Type 1 Diabetes with Insulin [§] vs. Type 2 Diabetes with Insulin [§]		**Type 1 Diabetes with Insulin [§] vs. Type 2 Diabetes with Insulin [§]**	
p-ER +	**0.28 (0.08-0.95)**	**0.04**	0.27 (0.06-1.15)	0.08
EGFR +	0.79 (0.16-3.97)	0.77	1.04 (0.17-6.16)	0.97
p-ERK 1/2 +	0.61 (0.19-1.95)	0.41	0.69 (0.20-2.41)	0.56
p-mTOR +	0.68 (0.19-2.44)	0.55	0.76 (0.19-3.12)	0.71
IGF1R +	1.67 (0.35-1.88)	0.52	1.15 (0.21-6.22)	0.87
Premenopausal women with breast cancer and diabetes				
	Insulin [§] vs. No Insulin [†]		**Insulin [§] vs. No Insulin [†]**	
p-ER +	0.99 (0.41-2.38)	0.98	0.66 (0.25-1.74)	0.40
EGFR +	1.05 (0.25-4.36)	0.95	0.71 (0.16-3.23)	0.66
p-ERK 1/2 +	0.91 (0.39-2.16)	0.83	0.85 (0.34-2.09)	0.72
p-mTOR +	2.27 (0.92-5.61)	0.08	2.01 (0.78-5.17)	0.15
IGF1R +	**5.04 (1.40-18.17)**	**0.01**	**5.10 (1.36-19.14)**	**0.02**
Postmenopausal women with breast cancer and diabetes				
	Insulin [§] vs. No Insulin [†]		**Insulin [§] vs. No Insulin [†]**	
p-ER +	1.97 (0.62-6.24)	0.25	1.88 (0.58-6.09)	0.29
EGFR +	3.53 (0.88-14.12)	0.07	3.29 (0.80-13.56)	0.10
p-ERK 1/2 +	1.93 (0.68-5.50)	0.22	2.00 (0.69-5.79)	0.20
p-mTOR +	2.84 (0.85-9.53)	0.09	2.80 (0.83-9.47)	0.10
IGF1R +	1.10 (0.35-3.46)	0.87	1.08 (0.34-3.41)	0.90

Women with ER-positive breast cancer and diabetes				
	Insulin [§] vs. No Insulin [†]		**Insulin [§] vs. No Insulin [†]**	
p-ER +	1.20 (0.57-2.54)	0.63	1.17 (0.52-2.64)	0.70
EGFR +	9.86 (1.00-97.80)	0.05	8.00 (0.73-87.51)	0.09
p-ERK 1/2 +	1.15 (0.55-2.42)	0.71	1.15 (0.53-2.48)	0.72
p-mTOR +	2.48 (1.00-6.14)	0.05	2.37 (0.93-6.03)	0.07
IGF1R +	2.08 (0.66-6.47)	0.21	1.97 (0.61-6.32)	0.26
Women with ER-negative breast cancer and diabetes				
	Insulin [§] vs. No Insulin [†]			
p-ER +	0.45 (0.05-4.18)	0.48		
EGFR +	1.44 (0.34-6.09)	0.62		
p-ERK 1/2 +	2.06 (0.50-8.53)	0.32		
p-mTOR +	2.86 (0.63-12.92)	0.17		
IGF1R +	3.90 (0.91-16.80)	0.07		

* Logistic regression for tumor IHC marker as the dependent variable, with a negative staining of the tumor marker as reference category. If adjusted OR are not given, there is not enough power to perform adjusted regression analyses. [#] Women with diabetes treated with insulin analogues regardless the use of concomitant human insulin (n=22) or noninsulin antidiabetic drugs. [¥] Women with diabetes treated with human insulin only regardless the use of concomitant noninsulin antidiabetic drugs. [§] Women with diabetes treated with insulin (analogues) regardless the use of concomitant noninsulin antidiabetic drugs. [†] Women with diabetes treated only with diet and exercise and users of noninsulin antidiabetic drugs only. [#] Adjusted for menopause (pre/post) at breast cancer diagnosis and BMI closest measure prior to breast cancer diagnosis (continuous). Cases and controls were matched on age at breast cancer diagnosis. *p-ER= phosphorylated estrogen receptor, EGFR=epidermal growth =factor receptor, p-ERK= phosphorylated extracellular signal-regulated kinase, p-mTOR=phosphorylated mechanistic target of rapamycin, IGF1R=insulin growth factor 1 receptor, OR=Odds Ratio, CI=Confidence Interval.*

S1 Figure. Diabetes status stratified by pathology laboratories (%)



Diabetes status was not differentially distributed between laboratories (chi-square test; p=0.09)

5

Chapter 6

No evidence for strong associations of diabetes mellitus and insulin use with breast tumor expression profiles

Submitted for publication

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Summary

Purpose: Women with diabetes have an increased risk of breast cancer and subsequent worse prognosis. We investigated whether diabetes or insulin use are associated with different breast tumor expression profiles.

Methods: RNA was isolated from 271 invasive breast tumors of women with or without diabetes (2:1 frequency-matched on year of birth and age at breast cancer diagnoses), which were randomly selected from an existing Danish breast cancer cohort. RNA sequencing data of 252 breast tumors was used for investigating associations between diabetes/insulin treatment and gene expression, specifically of genes in insulin-related pathways; and the PAM50 gene classifier. We also compared gene expression among insulin users and we stratified for Estrogen Receptor (ER) status and menopause.

Results: Gene expression of breast tumors of women with diabetes did not differ compared to those of women without diabetes ($p > 0.99$); nor according to the PAM50 gene signature ($p > 0.55$). Among women with diabetes expression of insulin-related genes did not differ between tumors of women treated with or without insulin either ($p > 0.98$), nor between human insulin only and insulin analogue users ($p > 0.46$). Similar results were found in analyses of subgroups by menopause or ER.

Conclusions: Based on this study in Danish women, it seems unlikely that signaling pathways involved in breast tumor development are significantly different, or at least differences are very small, in women with and without diabetes. Our findings also suggest that exogenous insulin exposure is not an important driver of differential gene expression in breast tumors.

Introduction

Diabetes has been associated with increased breast cancer risk and worse prognosis after breast cancer diagnosis, though the mechanisms underlying these associations are unknown [1, 2]. The Insulin Receptor (INSR) and the Insulin Growth Factor 1 Receptor (IGF1R) pathway play important roles in both diseases [3]. There is some evidence that women with diabetes, treated with or without insulin, develop different tumor subtypes compared to women without diabetes [4-7].

Previous studies, based on immunohistochemical (IHC) measurements of protein expression, found that (premenopausal) women with diabetes developed more often hormone receptor negative tumors [4, 5, 8] and tumors that overexpress IGF1R [9]. Furthermore, insulin treatment was previously found to be associated with IGF1R expression and activation of phosphorylated mammalian target of rapamycin (p-mTOR) in our sample of women with diabetes [10].

Gene expression profiling has been indicated as a better and more in-depth reflection of tumor biology, and can be used to study over- or under-expression of specific pathways [11, 12]. Therefore, our aim was to explore whether breast tumors of women with diabetes have different tumor expression profiles compared to women without diabetes; and whether insulin use leads to different expression patterns among women with diabetes.

Methods

Study design, patient selection and data collection

Details on patient selection and methods of data collection have been described previously [4]. The study protocol was approved by the Science Ethics Committee of the Region Midtjylland in Denmark (M-20110198). In short, our study population consisted of 312 randomly selected breast cancer patients who were identified from the from the Danish Breast Cancer Cooperative Group (DBCG) Registry between 2000-2010 [13]. Women with preexisting diabetes were sampled in strata of age (≤50 and >50 years) at breast cancer diagnosis and 2:1 frequency-matched (to allow analysis by insulin treatment) on year of birth and age at breast cancer diagnosis to women without diabetes. Diabetes-status was based on medical diagnosis obtained from the Danish National Patient registry. Women treated with insulin (≥2 prescriptions cumulatively in the period up to one year prior to breast cancer diagnosis) and women never treated with insulin were identified from the Danish Register of Medicinal Products Statistics. We distinguished between human insulin only users and insulin analogue users regardless the use of concomitant human insulin. Women treated solely with insulin were considered patients with type 1 diabetes, if they had a recorded diagnosis of type 1 diabetes or they were ≤30 years at diabetes diagnosis. All other women with diabetes were categorized as having type 2 diabetes. Menopausal status was obtained from the DBCG databank and information on Body Mass Index (BMI) prior to breast

cancer diagnosis was retrieved from electronic medical records. ER, PR, HER2 status of tumors was revised using Tissue Micro Arrays [14].

Sample selection, preparation and processing

RNA was extracted from 4 x 5µm slides for 271 formalin-fixed, paraffin-embedded (FFPE) tumors with a tumor nuclei percentage of ≥40 (Figure S1). RNA extraction was performed using the "RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE tissue" (Ambion, art. nr. AM1975) following the manufacturer's instructions. Based on Nanodrop assessment sufficient quantity (>250 ng) was obtained for all 271 samples. For 252 samples sequencing libraries were generated using the degraded stranded RNA Access library preparation kit from Illumina (Figure S1). The fragment Distribution Values $(DV)_{200}$ were above the recommended value of 30 for 98% of the samples. Up to 12 libraries were pooled together for sequencing. Each pool was sequenced single read with 65 base pairs in one lane of the Hiseq2500 Machine. The samples were sequenced in 3 batches; there was no batch effect.

RNA sequencing data processing

Reads were aligned against the human transcriptome (hg38) using Tophat2 (Tophat version 2.1.0/ Bowtie version 1.0) [15]. Tophat was guided by a reference genome and a reference transcriptome; the latter was created using a GTF file downloaded from Ensemble version 77. Gene counts, the absolute number of reads per gene, were generated using Icount which is based on the HTSeq-count [16]. The strandedness of the fragments generated during the library preparation was taken into account for both the alignment and the determination of the gene counts.

Statistical analysis

To investigate differential expressed genes between breast tumors of women with diabetes or without diabetes, treated with or without insulin, we used package edgeR and Limma from Bioconductor in R. Diabetes (yes/no), insulin (yes/no) or insulin type (human vs. insulin analogues) was included as the independent variable in the model and the gene counts as the dependent variable. Only genes with more than 30 counts in at least 50 samples were included. We defined a subset of insulin-related genes; including the PI3K pathway (hsa04151), MAPK pathway (hsa04010) and insulin signaling pathway (hsa04910) [17] and the genomic profile of tumors in our study population was determined using the PAM50 gene classifier[18]. In additional analyses we adjusted for ER status and we stratified for ER and menopause since it is known that hormone receptors can influence cell signaling [3]. In order to correct for library size, the Voom function within the Limma package has been used, and a correction for multiple testing was applied using the Benjamini-Hochberg procedure; genes with a p-value < 0.05 were considered differentially expressed.

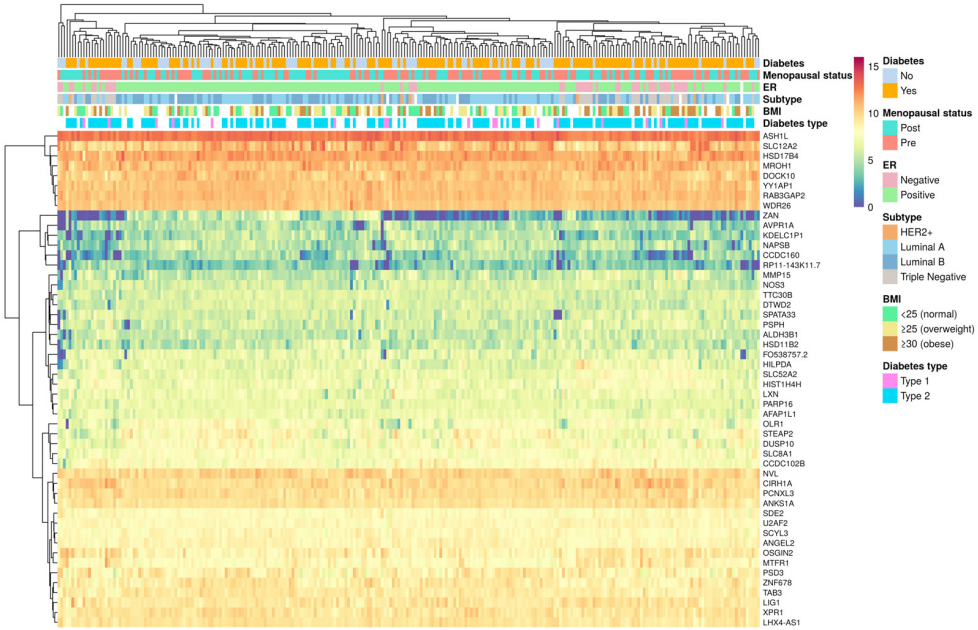
Heatmaps visualize the examined relationship and were generated using function pheatmap for the most highly variable genes. The absolute readcounts were normalized to 10 million reads per sample and $\log^2$ transformed. One pseudocount was added in order to avoid negative values.

Results

For 252 of 312 patients included (81%) RNA sequencing data were successfully generated (Figure S1). The average number of reads per tumor sample was 18 million; 72% of the reads were assigned to a gene and of those, 87% were mapped to protein coding genes. Of 252 included women, 171 patients had diabetes, of which the majority had type 2 diabetes (n=153), and 81 patients did not have diabetes. Patient and breast cancer characteristics at diagnosis have been published in detail previously [14]. Characteristics of the included women and whole study population were comparable (Table S1).

We found no association between diabetes-status and tumor gene expression. The expression of the 50 most significant genes are shown in a heatmap (Figure 1), however, after adjustment for multiple testing, all p-values became non-significant (p >0.99). Similar results were found in analyses of subgroups by menopause or ER-status. Among women with triple negative tumors, we found no significant differences in tumor gene expression for diabetes-status, although BEST3 was under expressed (log fold change=-1.86, p=0.07), and ZFAND4 (log fold change=1.28, p=0.08) and FDCSP (log fold change=5.59, p=0.08) were over expressed in women with diabetes compared to those without diabetes (Figure S2). Genes in the PAM 50 gene signature (for classification of breast cancer subtypes) were not differentially expressed between tumors of women with or without diabetes either (p >0.55). Breast cancer subtypes determined by IHC clustered together in the heatmap, but diabetes-status did not (Figure S3).

Figure 1. Unsupervised clustering of the most differentially expressed genes between tumors of women with or without diabetes

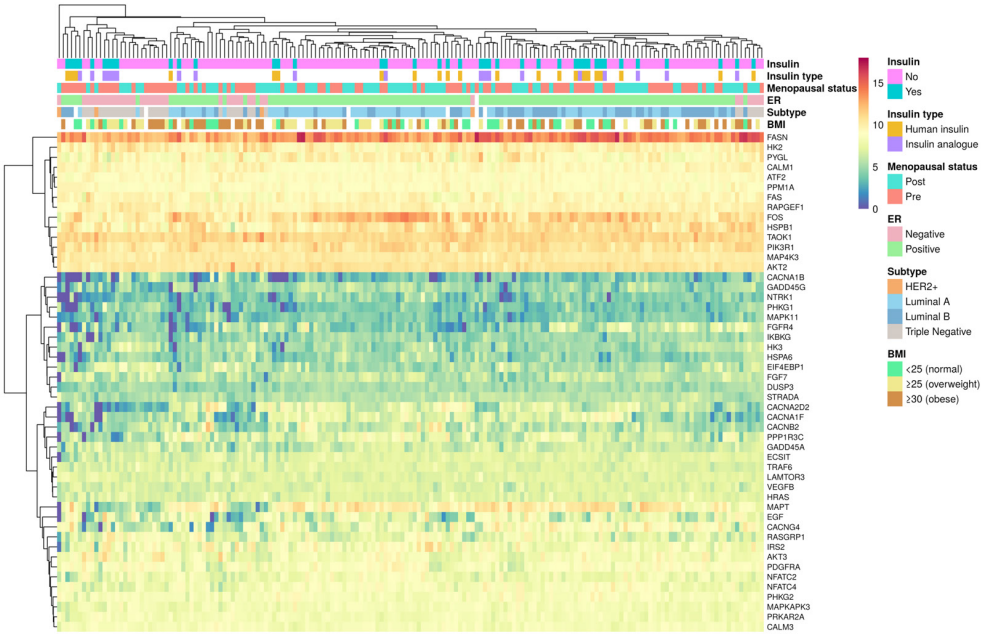


The heatmap visualizes the most differently expressed genes, based on the lowest p-values, for diabetes-status. However, after adjustment for multiple testing, all p-values became non-significant (p≥0.98).The dendrogram at the top shows the hierarchical clustering of the samples, which is accompanied by a panel of patient/tumor characteristics independent of the internal heatmap scales. The dendrogram at the side shows the clustering of the genes; gene expression is relative compared to the other genes (not row-scaled). *ER= Estrogen Receptor, BMI= Body Mass Index.*

Twenty four percent (n=41) of the women with diabetes were treated with insulin; 22 human insulin only and 19 insulin analogue users. Insulin use was not associated with tumor gene expression (p >0.98). Only NIPA1 was found to be overexpressed in insulin users compared to non-insulin users (log fold change=1.46, p=0.03) in women with ER-negative tumors. We did not find significant different expression of genes in insulin-related pathways between tumors of women treated with or without insulin after adjustment for multiple testing (Figure 2; p >0.98), nor between human insulin only and insulin analogue users (p >0.46). Analyses in subgroups by ER status gave similar results. Adjustment for ER status did not change the results in any of the analyses and tumors of women with similar BMI or diabetes type did not cluster together in the heatmaps either.

In Figure S4 the expression of genes that play a key role in insulin-signaling or might interact with insulin, such as ESR1, are presented among women with diabetes treated with or without insulin. Unsupervised clustering divided tumors that overexpress ESR1 and IGF1R and those that do not; the first co-clustered with ER-negative tumors. The clustering was independent of insulin use.

Figure 2. Unsupervised clustering of the most differentially expressed insulin-related genes between tumors of women treated with or without insulin



The heatmap visualizes unsupervised clustering of the expression of insulin-related genes with the lowest p-values between insulin and non-insulin users. However, after adjustment for multiple testing, all p-values became non-significant (p≥0.97). The dendrogram at the top shows the hierarchical clustering of the samples, which is accompanied by a panel of patient/tumor characteristics independent of the internal heatmap scales. The dendrogram at the side shows the clustering of the genes; gene expression is relative compared to the other genes (not row-scaled). *ER= Estrogen Receptor, BMI= Body Mass Index.*

Discussion

Overall, we found no evidence that women with diabetes develop breast tumors with different expression profiles compared to women without diabetes. Furthermore, expression of genes in insulin-related pathways did neither differ between tumors of women with diabetes treated with or without insulins, nor between human insulin and insulin analogues.

Our study consists of a sufficiently large sample of randomly selected women with breast cancer, with or without a medical diagnosis of diabetes, of which the tumors were RNA-sequenced. We derived history of treatment with insulin, which was available at least 5 years prior to breast cancer diagnosis, from prescriptions records and we had information on potential confounders and effect modifiers. All tumors were prepared and analyzed in a central experienced genomic facility to assure high data quality.

The main limitation was that RNA sequence data were generated using 6 to 16-year old FFPE tumor tissue collected from several pathology departments in Denmark. The percentage of reads that were assigned to a gene slightly decreased with time since storage of the tumor tissue in the archives and varied between pathology departments. However, age of the sample and pathology department did not influence clustering of the samples. More importantly, around 14 million reads (standard deviation of 4 million) per sample were aligned to the transcriptome of which 87% were aligned to protein coding genes, which should be sufficient to perform proper gene expression analyses [19]. Our results were validated: genes known to be overexpressed such as GAPDH and ACTB (positive controls) were overexpressed in all samples, expression of previously stained proteins (e.g. ER, IGF1R) correlated with gene expression, and clustering of our samples according to the PAM50 gene signature was concordant with breast cancer subtypes defined by IHC (Figure S3). Moreover, earlier publications have affirmed the successful use of archival FFPE tumor tissue for RNA sequencing [20, 21].

It has been shown that certain conditions, such as BMI, can lead to differences in breast tumor gene expression [22]. However, studies investigating the association between diabetes or insulin treatment and tumor gene expression were lacking. One study investigated breast tumor gene expression of the IGF1, IGF1R and the IGFBP3 in women with or without type 2 diabetes (n=40) and found no association between tumor gene expression of these proteins and diabetes [23]. Prior to the start of our study, we considered a shared genetic etiology between type 2 diabetes and certain breast cancer subtypes with specific gene expression profiles. However, based on recent studies there is little evidence for any shared hereditary genetics between diabetes and breast cancer [24, 25]. Additionally, we considered that insulin analogues may increase risk of breast cancer because of their potential impact on tumor progression through e.g. the insulin-like growth receptor pathway [3]. However, a recent meta-analysis reported no association between insulin analogues and breast cancer risk [26]. The results of our study that tumor genotype of women with diabetes, treated with or without insulins do not differ from women without diabetes, are in line with these findings.

Though we previously found that tumor ER protein expression was negatively associated with diabetes and IGF1R protein expression was positively associated with insulin treatment in premenopausal women [10], we did not confirm this considering the RNA expression. This is not necessarily contradicting as protein expression not only depends on mRNA concentration but also on the translation efficiency and protein degradation [27].

In conclusion, we found no association between tumor gene expression and diabetes or insulin use in Danish women with breast cancer.

Acknowledgement

We acknowledge the Departments of Pathology in Denmark for retrieving the tumor tissues and the Danish Breast Cancer Cooperative Group and Statistics Denmark for access to their databases. We thank Peer Christiansen, president of the Danish Breast Cancer Society, for helping with the acquisition of the data and we thank Jacob Starup Linde for his contribution to the data collection from the electronic medical records. We acknowledge the NKI-AVL Core Facility Molecular Pathology & Biobanking for preparation of the RNA samples and the Genomic Core Facility for RNA sequencing of the breast tumors.

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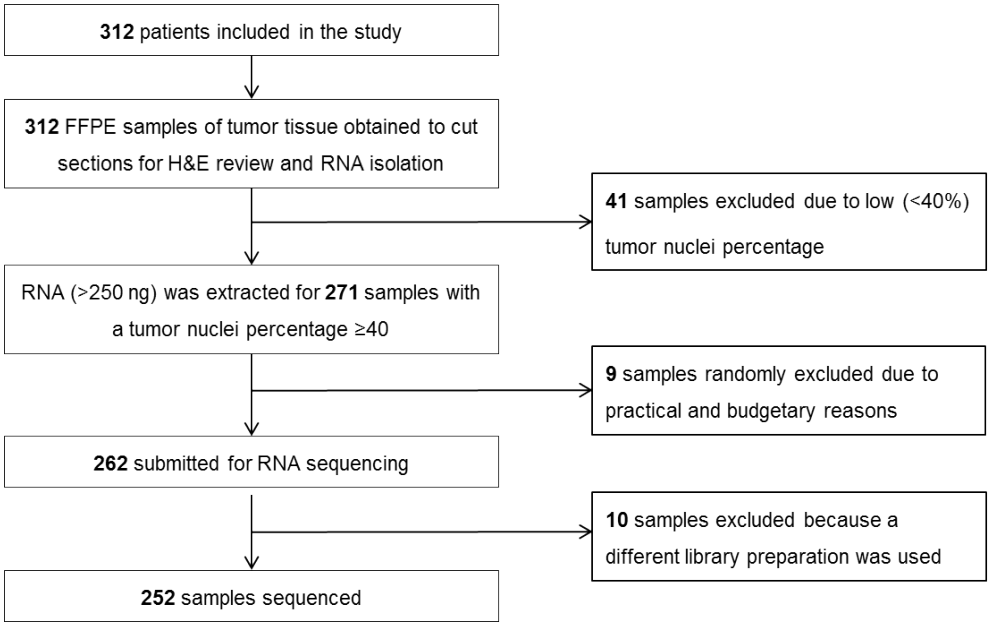
Supplementary material

Figure S1. Flow chart of Formalin-Fixed Paraffin Embedded primary breast tumor sample selection for RNA sequencing



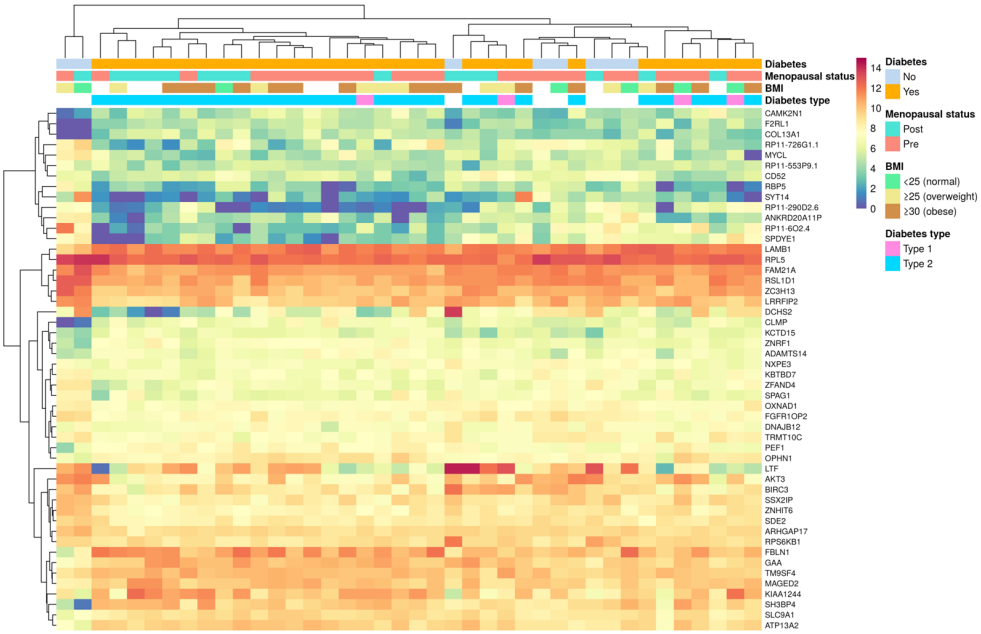
The most representative tumor block was selected for each patient. The percentage of tumor nuclei was determined by a breast pathologist and was revised by another pathologist. FFPE: Formalin-Fixed Paraffin Embedded, H&E: Hematoxylin and Eosin

Table S1. Characteristics of breast cancer patients in the whole and sequenced study population

	Whole study population (n=312)	**Sequenced subset** (n=252)
Age, median (IQ range) [a, b]		
≤ 50 years	47 (43-50)	47 (43-50)
> 50 years	67 (61-74)	68 (62-75)
BMI in kg/m^2, median (IQ range) [c]		
Premenopausal women	26.6 (23.5-31.2)	26.8 (23.9-31.6)
Postmenopausal women	28.0 (24.1-30.4)	27.0 (24.1-30.0)
Year of breast cancer diagnoses, median (IQ range) [a]	2007 (2004-2009)	2006 (2004-2008)
	% (n)	**% (n)**
Menopausal status [b]		
Pre	51.0 (159)	50.4 (127)
Post	49.0 (153)	49.6 (125)
Diabetes	67.6 (211)	67.9 (171)
Diabetes Type		
Type 1	11.8 (25)	10.5 (18)
Type 2	88.2 (186)	89.5 (153)
Insulin treatment	25.1 (53)	24.0 (41)
Morphology		
Ductal	74.0 (231)	75.4 (190)
Lobular	8.7 (27)	8.7 (22)
Other	17.3 (54)	15.9 (40)
Grade		
Grade 1	19.9 (60)	18.0 (44)
Grade 2	40.7 (123)	41.2 (101)
Grade 3	39.4 (119)	40.8 (100)
ER		
Positive	80.4 (250)	80.9 (203)
Negative	19.6 (61)	19.1 (48)
PR		
Positive	67.0 (209)	68.4 (171)
Negative	33.0 (103)	31.3 (79)
HER2		
Positive	87.1 (270)	87.6 (219)
Negative	12.9 (40)	12.4 (31)

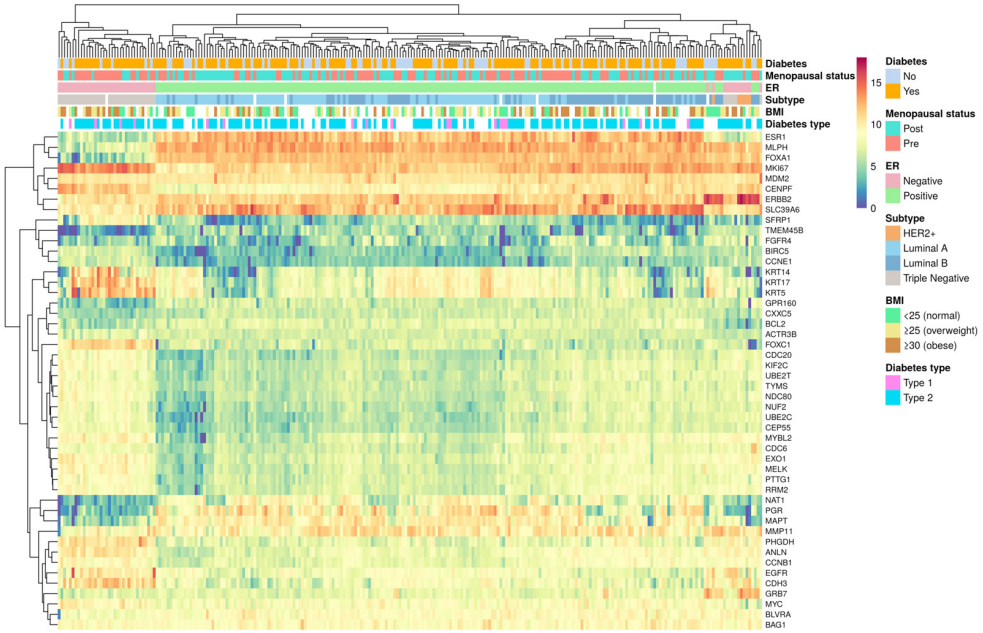
Comparison of the clinic-pathologic and treatment characteristics of the whole cohort of patients and the sub-study cohort analysed in this project. [a] Matching variable, [b] At breast cancer diagnosis, [c] Closest measure prior to breast cancer diagnosis. Missing values are not shown, therefore the sum of the categories does not add up to the total number of patients for grade, ER and HER2. *IQ=interquartile range, BMI=Body Mass Index, ER= Estrogen Receptor, PR=Progesterone Receptor, HER2=Human Epidermal growth factor Receptor 2.*

Figure S2. Expression patterns of the most differentially expressed genes between triple negative tumors of women with diabetes or without diabetes



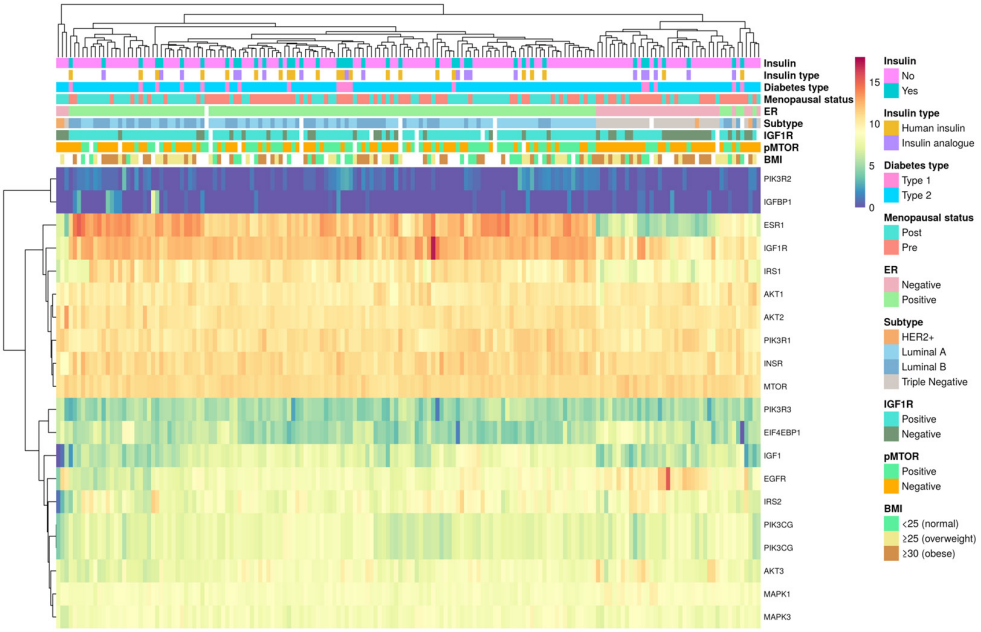
The heatmap visualizes the most differently expressed genes, based on the lowest p-values, for diabetes-status among women with a triple negative tumor, However, after adjustment for multiple testing, all p-values became non-significant ($p \geq 0.07$).The dendrogram at the top shows the hierarchical clustering of the samples, which is accompanied by a panel of patient/tumor characteristics independent of the internal heatmap scales. The dendrogram at the side shows the clustering of the genes; gene expression is relative compared to the other genes (not row-scaled). BMI= Body Mass Index.

Figure S3. Expression of genes in the PAM50 signature among breast tumors of women with or without diabetes



The heatmap visualizes the expression of the PAM50 genes among breast tumors of women with and without diabetes. The dendrogram at the top shows the hierarchical clustering of the samples, which is accompanied by a panel of patient/tumor characteristics independent of the internal heatmap scales. The dendrogram at the side shows the clustering of the genes; gene expression is relative compared to the other genes (not row-scaled). *ER= Estrogen Receptor status, BMI= Body Mass Index*

Figure S4. Expression of a selection of insulin-related genes in breast tumors of women with diabetes treated with or without insulin



The heatmap visualizes the expression of a selection of insulin-related genes among breast tumors of women with diabetes. The dendrogram at the top shows the hierarchical clustering of the samples, which is accompanied by a panel of patient/tumor characteristics independent of the internal heatmap scales. The dendrogram at the side shows the clustering of the genes; gene expression is relative compared to the other genes (not row-scaled). *ER= Estrogen Receptor status, BMI= Body Mass Index, IGF1R= Insulin Growth Factor 1 Receptor, pMTOR= phosphorylated mechanistic target of rapamycin.* IGF1R and pMTOR were immunohistochemically stained as described previously [4].

6

Chapter 7

General discussion

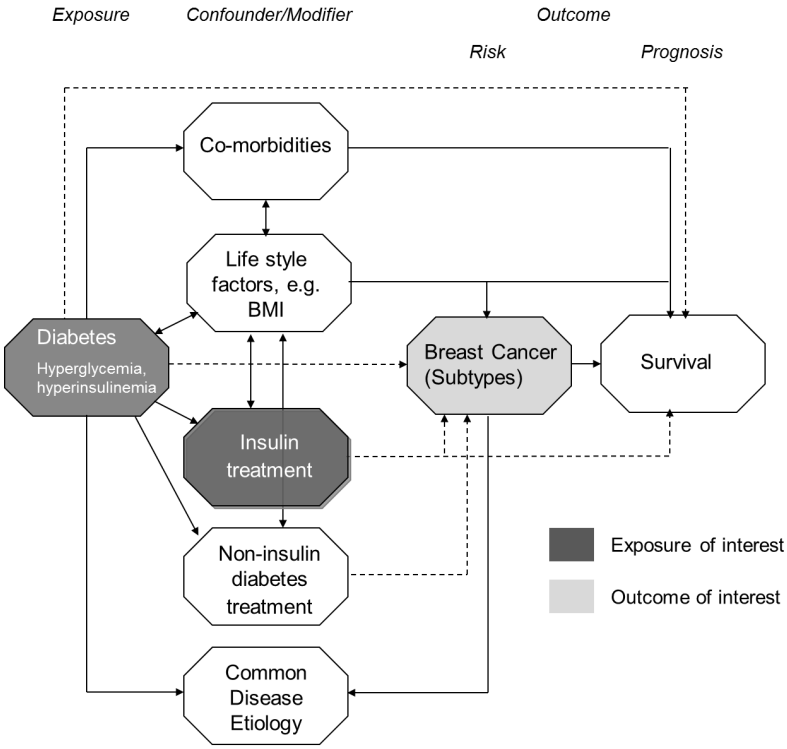
The general aims of this thesis were to assess whether diabetes, and specifically insulin treatment, is associated with breast cancer development as well as with different breast cancer subtypes, and to investigate potential mechanisms involved. In this concluding chapter we discuss the main findings and we interpreted them in a broader context. The methodological challenges of performing observational research and the strengths and limitations of our studies are discussed. Finally, recommendations for future research and clinical implications are given.

Main findings in context of other literature

Breast cancer risk in women with diabetes

Several studies reported that women with (type 2) diabetes are at increased risk of developing breast cancer [1-7]. Diabetes and breast cancer have a partial shared etiology, mostly related to hormone and growth factors. Consequently these diseases share several risk factors including obesity (high BMI) and older age (Figure 1) [8]. The prevalence of cancer- and diabetes-promoting factors, such as obesity and a sedentary lifestyle have increased over the last decades. We wondered whether incidence rates of breast cancer among women with and without diabetes increased over time. We found in the Clinical Practice Research Datalink (CPRD) that incidence rates of breast cancer among women with type 2 diabetes in the UK remained stable between 1989-2012, the incidence rate was approximately 150 per 100,000 women years (chapter 2). Unexpectedly the breast cancer incidence in women with diabetes was comparable to women without diabetes. This difference in results might be caused by differences in classification of diabetes mellitus. Based on our study results and studies that found a small increased risk for breast cancer in women with diabetes [1-7], there is no need for a different (e.g. intensified) screening approach for breast cancer among women with type 2 diabetes. To further understand how women with diabetes might have a higher risk for breast cancer we evaluated whether insulin and insulin analogues might contribute to an increased risk of breast cancer in women with diabetes.

Figure 1. Schematic overview of the complex environment in which the association between diabetes, insulin use and breast cancer is studied.



Breast cancer risk after insulin use for treatment of diabetes

Based on the review of published *in vitro*, *in vivo* and human evidence (epidemiological studies as well as randomized clinical trials) (chapter 3), we concluded that there is no compelling evidence that treatment with insulin or insulin analogues increases breast cancer risk among women with diabetes. Though cautious interpretation of the results is necessary as a result of methodological shortcomings of the included studies as discussed in chapter 3, our conclusions were confirmed by recently published epidemiological studies [9, 10]. Those recently published studies had a proper design and a large sample size and included new insulin users with longer duration (median 5 years) of exposure; risk of developing breast cancer was not increased in insulin analogue users compared to human insulin users [9, 10]. Two other studies examined breast cancer risk in insulin users (long- and short-acting insulin and human insulin) compared to women never treated with insulin, and also found no increased risk of breast cancer associated with insulin use [11, 12]. Although prolonged use (>5 years) of human insulin was associated with a 20% increased risk of breast cancer [12]. Similar to insulin, metformin and other non-insulin antidiabetic drugs such as sulfonylureas and thiazolidinediones have been shown to only slightly reduce, if at all, breast

cancer risk [13-16]. In conclusion, there is very little indication that human insulin, insulin analogue treatment or other non-insulin antidiabetic medication is associated with risk of breast cancer.

Breast cancer subtypes in insulin and non-insulin treated women with diabetes

Despite the lack of association between insulin (analogue) use and breast cancer risk it is still possible that these drugs influence the progression of breast cancer. Therefore, we studied whether insulin and non-insulin treated women with diabetes, develop specific breast cancer subtypes compared to women without diabetes. This is a relevant question since breast cancer subtype is an important determinant of prognosis [17]. Studies about such associations were very scarce [18-21], due to the comprehensive data needed to perform such studies. However, no association between diabetes or insulin treatment with clinicopathological subtypes existed in our study (chapter 4). Only premenopausal breast cancer patients with diabetes tended to develop breast tumor that do not express hormone receptors and basal-like tumors, which are typically associated with poor prognosis compared to premenopausal women without diabetes. However, analyses of expression of genes in the PAM 50 gene signature [22], used for classification of breast cancer subtypes, did not confirm these findings (chapter 6). The few studies published that examined breast cancer characteristics in women with diabetes treated with or without insulin [18-21, 23, 24], reported results that are consistent with ours. The studies that stratified for menopausal status, also reported that premenopausal women with diabetes developed more often tumors that were hormone receptor negative [19, 21]. In contrast to our findings, an ongoing study in the Netherlands Cancer Registry-PHARMO Database Network cohort with a larger sample size, indicated that women treated with insulin (analogues) (n=149) are at increased risk of developing more aggressive breast tumors (more advance tumor stage, higher grade, more luminal B vs Luminal A tumors) than women using oral antidiabetic treatment (n=289) or no antidiabetic treatment (n=596) [25]. A limitation of this study is that tumor characteristics were evaluated in different laboratories and by different pathologists since these were collected from the Cancer Registry [26, 27].

We could not disentangle whether the development of tumors that lack expression of hormone receptors in premenopausal women with diabetes was due to hyperglycemia, hyperinsulinemia, side effects of diabetes treatment or risk factors such as obesity, or a combination of those (Figure 1). However, insulin treatment or other types of treatment such as metformin, were not associated with the development of a particular clinicopathological subtype (chapter 4). Since we only found associations in premenopausal women, differences in levels of BMI-related and reproductive hormones, i.e., factors related to menopausal status, such as insulin, estrogen and adipokine, may play a role in the specific tumor subtype development [28]. Further studies would be needed to answer these questions. Overall, based on the limited amount of data published, there is no compelling evidence that women with diabetes, treated with or without insulin, are at risk of developing more aggressive or less-treatment responsive breast cancer subtypes.

Tumor protein and gene expression in women with diabetes and in insulin users

Gene expression profiles of tumors of women with diabetes did not differ from those of women without diabetes. Expression of genes in insulin-related pathways did neither differ between tumors of women with diabetes treated with or without insulins, nor between human insulin and insulin analogues (chapter 6). To our knowledge, no other studies examined gene expression profiles of breast tumors of women with diabetes, though several investigated the association between gene expression and breast cancer prognosis [17, 22, 29]. Based on protein expression analyses we found that IGF1R and p-mTOR were more often expressed among insulin users compared to non-insulin using diabetes patients (chapter 5). However, altogether it is unlikely that signaling pathways involved in breast tumor development are significantly different, or at most differences are very small, in women with and without diabetes. Summarizing, it seems that exogenous insulin exposure is not an important driver of tumor gene expression, which is in line with our previous conclusion that insulin (analogues) do not increase risk of breast cancer.

Potential mechanisms and etiology of increased breast cancer risk (and progression) in women with diabetes

Over the last years, several preclinical and epidemiological studies have investigated potential molecular mechanisms related to diabetes itself that might increase breast cancer risk [6, 28, 30-38]. Others addressed the association between insulin and non-insulin antidiabetic drugs or shared genetic risk factors and breast cancer risk in women with diabetes [39, 40]. This complex environment is presented in Figure 1.

Experimental data supports that insulin treatment is involved in tumor progression rather than tumor initiation (chapter 3). The most plausible hypothesis for breast tumor progression in insulin-treated women with diabetes, is through phosphorylation of INSR or IGF1R, caused by insulin binding, resulting in upregulation of mitogenic signaling cascades (MAPK or PI3K) [41-45]. The results of a recently published *in vivo* study in a human relevant mammary gland mouse model substantiate these findings. They found that gene expression profile of IGF1R induced tumors showed an increased and sustained proliferative and invasive profile. This indicates that the decreased tumor latency time in IGF1 and insulin AspB10 treated mice is related to changes related to tumor progression rather than increased tumor initiation [46]. In contrast, another study in a type 2 diabetes mouse model showed that insulin analogues did not increase tumor growth compared to vehicle treatment [47]. Furthermore, randomized clinical trials were negative for increased breast cancer risk for insulin glargine or insulin detemir compared to human insulin or standard care [48-51]. Importantly, though *in vitro* and *in vivo* studies showed mitogenic potential of insulin (analogues) via upregulation of the INSR or IGF1R (chapter 3), these experimental findings have not been confirmed in the human setting (chapter 5 and 6). This might be due to exposure at supra-physiological concentrations, the use of tumor cell lines instead of normal mammary cell lines, but also due to tissue specific responses, e.g. the rapid enzymatic conversion of glargine

in vivo into two metabolically active compounds with low mitogenic potential. Additionally, in *in vitro* and *in vivo* studies, compounds with high affinity towards the IGF1R, such as IGF1 or insulin AspB10, were used that are not available in clinical practice. It could also be that the effects are not large enough to be clinically relevant, particularly not in an environment with many potentially modifying factors (Figure 1).

It has also been suggested that insulin may enhance estrogen production. As a result estrogen levels might be increased in women with diabetes, which are considerable potentially carcinogenic conditions for particularly the breast [28]. Interactions between insulin and estrogen could act synergistically during tumor development [28, 30, 35] and therefore, the promotion of tumor growth upon insulin exposure may vary for different breast cancer subtypes. This may suggest that women with diabetes develop different breast cancer subtypes (more ER-positive tumors) than women without diabetes, but based on existing evidence this is unlikely.

Based on our results presented in chapter 2 it is less certain that women with diabetes have an increased breast cancer risk. When this increased risk exists, it is highly unlikely that diabetes treatment contributes to this risk and it might be that underlying factors related to diabetes itself contribute (Figure 1). Our study design (chapter 4, 5, 6) did not allow to study potential causal factors such as hyperinsulinemia or hyperglycemia. Others showed that chronic inflammation associated with diabetes (hyperglycemia) and obesity promotes oxidative stress, which may create a microenvironment favorable to tumor development [33, 34, 37]. Insulin resistance and hyperinsulinemia might also favor breast tumor growth via the INSR and IGF1R (chapter 1). Although we did not have data on endogenous insulin levels, we did not observe differences in expression of insulin-related proteins between women with type 1 and type 2 diabetes and without diabetes. There is some inconsistency between results of epidemiological studies, but overall it seems that hyperinsulinemia in women without diabetes contributes to the risk of breast cancer in postmenopausal women [36, 38, 52, 53]. Important to note is that obesity and diabetes are strongly interrelated and that both may also contribute to an increased risk of breast cancer. Studies in which type 2 diabetes was associated with increased breast cancer have shown that adjustment for BMI did not modify the association between diabetes and breast cancer risk [1, 2]. Furthermore, a recent study indicated that hyperinsulinemia may be more biologically relevant to the development of breast cancer than obesity per sé [54]. Further studies are needed to evaluate the independents effect of hyperinsulinemia, obesity and diabetes on breast cancer risk.

In general, it is believed that breast cancer in women with diabetes is diagnosed at an advanced stage compared to women without diabetes [19, 23, 55-57]. Although we did not observe strong evidence that diabetes or insulin treatment is associated with tumor size, number of positive lymph nodes or grade (chapter 4), this is not necessarily contradicting since we only included women with non-metastasized cancer. Moreover, overall mortality after breast cancer diagnosis in women with pre-existing diabetes was reported to be higher compared to women without diabetes [55, 56, 58-61]. However, prior to the start of this thesis it was unclear whether this worse

overall mortality was really related to breast cancer itself, i.e. whether there was worse breast cancer specific survival. Although there is significant heterogeneity between studies, based on two recently published meta-analyses there is an indication that diabetes is a risk factor for breast cancer specific mortality [5, 62]. Whether insulin use contributes to the worse breast cancer specific survival remains unclear [63-65]. Given the lack of evidence for the development of differential breast tumor subtypes in women with diabetes, this worse survival is probably not, or only marginally, mediated by the development of more aggressive breast cancer subtypes. However, further research in larger populations, stratified for menopausal status, is warranted.

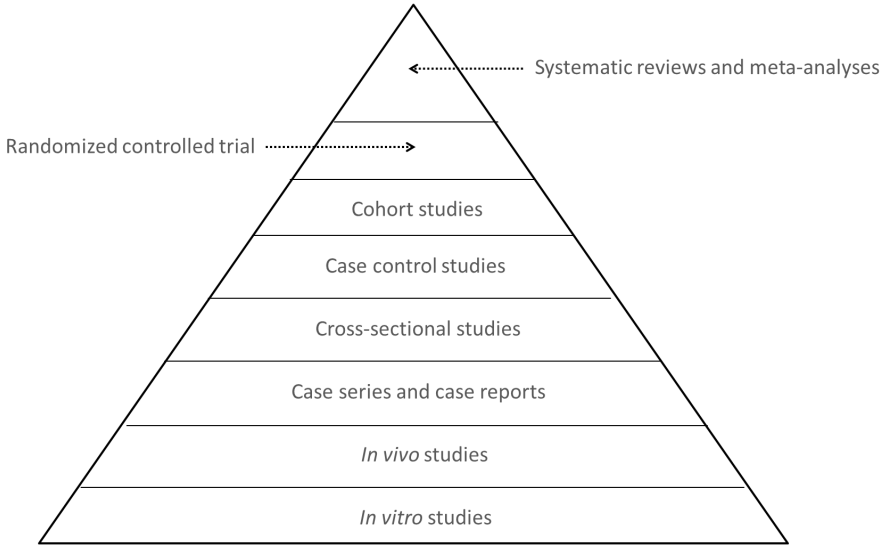
While efforts of scientists have contributed to the understanding of the role of diabetes and related metabolic alterations in the development of breast cancer, there is still no consensus on the underlying causal factors. Since diabetes medication does not increase the risk of breast cancer and based on recent studies there is little evidence for any shared hereditary genetics between type 2 diabetes and breast cancer [39, 40], it seems more likely that women with diabetes might have a higher risk of developing breast cancer due to factors that are associated with underlying biological factors such as obesity and hyperinsulinemia. Further molecular epidemiological studies are necessary to elucidate the complex interrelations between mediating pathways of breast cancer promotion in women with diabetes.

Strengths, limitations and methodological challenges

Unique combination of several levels of evidence

The work described in this thesis provides a unique combination of *in vitro*, *in vivo* and epidemiological data examining the role of diabetes and insulin treatment on breast cancer risk and breast cancer subtypes. We are the first to perform such an extensive study into the role of diabetes and insulin treatment on breast tumor etiology in humans. The qualitative and quantitative evaluation of all published literature on the carcinogenic potential of insulin (analogues) *in vitro* and *in vivo* and human studies (chapter 2) provided a good starting point to determine the knowledge gaps. The hypotheses that were generated from published *in vitro* and *in vivo* studies were tested in the clinical setting and contributed to the understanding and interpretation of our findings in human breast tumors (chapters 4, 5 and 6). While interpreting the findings, we kept in mind the strength of the different types of scientific studies as described in the levels of the evidence-based pyramid (Figure 2). Studies in humans are the gold standard for evaluating evidence of exposure and disease.

Figure 2. Evidence based pyramid of *in vitro, in vivo* and human studies



Access to large and detailed databases

A strength of the studies presented in this thesis is that breast cancer patients were randomly selected from a nation-wide hospital based cohort by the Danish Breast Cancer Cooperative Group (DBCG) to prevent selection bias, and women with diabetes were matched to women without diabetes by year of birth and age at diagnosis (chapter 4, 5 and 6). The patients in this cohort have been considered to represent the Danish breast cancer population [66]. We were fortunate to have access to comprehensive biobanks and databases available in Denmark. Scandinavian countries are unique in the storage of clinical data on medication use and medical diagnosis as well as socioeconomic data in national registries since the 1980s. We obtained medical histories of study participants, including the medical diagnosis of diabetes, the date of diagnosis and the type of diabetes by linkage with the National Registry of Patients. We had access to very detailed medication histories, at least five years prior to breast cancer diagnosis, from prescriptions records by linkage with the National Registry of Medicinal Product Statistics, including the duration and type of antidiabetic treatment. BMI and other lifestyle factors were manually collected from medical records. Because we had extensive data on variables that were correlated with BMI such as income, education and cardiovascular disease, we were able to impute the remaining missing data. In addition, due to oversampling of young breast cancer patients, we could examine the association between diabetes/ insulin treatment in both pre- and post-menopausal breast cancer patients.

Availability of tumor tissue and high quality data due to central revisions

In the patient selection, we included the availability of tumor tissue as an inclusion criterion. We assured good quality tumor data by relying on expert breast pathologists for the retrieval, review, staining, and scoring of the breast tumors for clinical and insulin-related immunohistochemical markers. All immunohistochemical stainings within one study were validated and performed in one center and scored by the same breast pathologist, to prevent inter-laboratory and inter-observer variability [26, 27]. To generate the tumor gene expression data, all tumors were prepared and analyzed in a central experienced genomic facility to assure quality of the data.

Samples size

One of the main limitations of our study was the sample size. Originally we intended to retrieve 600 tumor samples from the Netherlands and Denmark. Unfortunately, the Dutch cohort was cancelled because we were unable to link the Dutch breast cancer cohorts from the Netherlands Cancer Institute to prescription records within a reasonable timeframe. Although we identified 3,047 women with diabetes among the 43,701 women that diagnosed with breast cancer in Denmark, we could not enlarge our sample size due to limited time and budget constraints. Although data on grade and some hormone receptor markers are available (or at least ER, PR, and HER2 status) in the cancer registry to perform a larger study, these data were only retrieved for the patients selected in our study. Importantly, we had observed that these data were very incomplete and scored heterogeneously by different pathologists. Since we had to half our samples size, our study was only sufficiently powered to detect large differences between breast cancer subtypes, e.g. 80% versus 60% ER-positive tumors, in women with and without diabetes and therefore, subtle differences may not have been detected. We included twice as many women with diabetes as women without diabetes to allow analyses by insulin treatment. Although we had very detailed information on insulin treatment (dose and duration), we had limited power for analyses among insulin users. We especially had limited power to investigate different insulin types, due to the reduction in sample size, and due to an unexpected large number of women (40%) with clinically diagnosed diabetes that were not treated with antidiabetic treatment.

Patient selection, confounding and effect modification

Another important limitation was that at the time of patient identification only age, year of breast cancer diagnosis and diabetes were available. Data on other important variables such as type of diabetes, menopausal status and BMI only became available after the tumor block selection due to cost of data linkage and retrieval and time of manual data collection from medical records. As a result we were not able to exclude women with type 1 diabetes and we were not able to match women with and without diabetes by BMI, an important potential effect modifier.

Potential confounding bias and effect modification are known limitations in observational research since other risk factors are usually not equally distributed between the exposed and

non-exposed group. As we explained in chapter 1, diabetes and breast cancer share many risk factors, which are generally interrelated, such as BMI. Furthermore, there are several other factors which may be the driving forces between the increased risk of breast cancer or specific breast cancer subtypes among women with diabetes such as hyperinsulinemia and hyperglycemia, and these factors vary dependent on the type of diabetes and the severity of diabetes (Figure 1). For example, the severity and type of diabetes influences exposure to type of treatment, endogenous levels of glucose and insulin, as well as the duration of exposure. This makes it extremely hard to disentangle the true association between diabetes, insulin treatment and breast cancer.

Due to the cross-sectional/retrospective design of the studies included in this thesis, it was difficult to collect data on potential confounders/effect modifiers, and therefore residual confounding may still be present. We lacked data on reproductive factors and biomarkers of control of diabetes such as HbA1c or c-peptide were incomplete or not available. Lifestyle factors such as BMI were collected from medical records, which have its obvious restriction due to missing data and the time of measurement (though as argued above, we were able to impute BMI). For BMI we used the closest measure prior to breast cancer, while it has been reported that cumulative duration of exposure to BMI is a better predictor for breast cancer risk[67]. Even so, in our analyses duration of diabetes, BMI and other diabetes medication, did not seem to affect the associations between diabetes or insulin treatment with breast cancer risk, breast cancer subtypes or insulin signaling pathways. Therefore, we do not believe that our conclusions would have been substantially different if we would have had more complete data.

Variation and misclassification of diabetes and insulin exposure

The definition of the exposure of interest and the definition of the exposure comparison is crucial for the interpretation and extrapolation of scientific findings. Differences in diabetes ascertainment, but also differences in exposure classification of insulin users (chapter 3), between studies make results hard to compare and might partly explain observed discrepancies (chapter 2). For example in chapter 2 we used anti-diabetic medication as a proxy to identify women with diabetes while others defined diabetes based on self-report, blood glucose levels or discharge diagnosis [1, 2]. One can imagine that studies that included women hospitalized for their diabetes possibly suffered from more advanced disease compared to women with diabetes included in the study presented in chapter 2. In the studies described in chapters 4, 5 and 6, women with diabetes were identified based on medical diagnosis. Forty percent of these women with diabetes were treated with diet and physical activity only, a group of patients that was not included in the study of chapter 2. Although medical diagnoses are a reliable source for the definition of a disease, we might have misclassified some women in our reference group (chapters 4, 5 and 6), since many women are living with undiagnosed type 2 diabetes [68]. Therefore, the results of chapter 2 and chapter 4, 5 and 6 might have been biased towards the null.

Another source of variation is the in- or exclusion of women with type 1 and type 2 diabetes. As explained in chapter 1, diabetes type 1 and type 2 are diseases with their own entity and treatment regimen, and therefore the association with breast cancer might be different. Ideally one would include either women with type 1 diabetes, or women with type 2 diabetes. Although we did not observe significant differences in associations between women with type 1 and type 2 diabetes and breast cancer subtypes/genotype, we only included 25 women with type 1 diabetes. As the prevalence of type 1 diabetes is relatively rare, it is hard to include sufficient women with type 1 diabetes to have enough power to study the relation with breast cancer risk or breast cancer subtypes.

The time between exposure and outcome is another point that needs some attention. The mean time between diabetes and breast cancer diagnosis was 8.9 years and the mean time between first insulin prescription or first non-insulin antidiabetic prescriptions and breast cancer diagnosis was respectively 8.4 years and 5.5 years. Though, we cannot guarantee the sequence of events (insulin exposure and subsequent tumor promotion) because of the potential lag time in the detection of the tumor. However, tumor size (an important factor for detection) was not related to diabetes status or insulin exposure, so it is unlikely that the associations we observed were due to reverse causation. Additionally, it is questionable whether a relative short time of exposure will have effect on breast cancer etiology.

Use of formalin-fixed, paraffin-embedded (FFPE) tissue in molecular epidemiological studies

Another challenge is the use and collection of FFPE tissue for molecular epidemiological studies. Tumor tissue block were collected from several pathology laboratories across Denmark and as a result the embedding and storage of tissue blocks may have been different between laboratories. Thig might have affected the results presented in this thesis. However, this would only have confounded the analyses if diabetes status or insulin use would have been differentially distributed between laboratories or years of diagnosis, and this was not the case. Use of FFPE tissue has also other limitations, since the embedding in paraffin causes degeneration/fragmentation of the tissue. However, the use of FFPE for immunohistochemistry and RNA sequencing has been proven to be successful by others [69-71] and we only used techniques/methods/kits/antibodies that were validated for FFPE tissue.

Clinical implications and suggestions for future research

There are no direct clinical implications regarding the treatment of women with diabetes resulting from this thesis since we can conclude that insulin and insulin analogues treatment do not increase risk of breast cancer. Treatment of diabetes is essential as improving metabolic control to approach normal glycaemia greatly benefits long-term prognoses and insulin treatment in type 1 diabetes is lifesaving [68]. We want to emphasize the importance of proper evaluation of carcinogenic effects of a drug. In case of insulin analogues, a possible safety hazard was

communicated based on studies with methodological limitations. We are glad that, due to proper evaluation, (long-term) safety of insulin and insulin analogues is established.
There is some indication, especially among premenopausal women, that diabetes and/or insulin is associated with the development of poor prognosis tumors (chapter 4 and 5) [19, 21, 72], but all of these studies were relatively small. Therefore a meta-analysis of clinicopathological subtypes in women with diabetes should be performed. In order to do so, there is a need for (small) high quality studies investigating these potential associations, preferably stratified for menopausal status. In general, more research is needed to investigate breast cancer risk and breast cancer etiology in women with type 1 diabetes. Diabetes and insulin are one of the many factors that may play a role in the etiology of development of breast cancer subtypes, and associations between subtypes and other risk factors such as BMI and parity are also still inconclusive [73-75].
Since it is still uncertain whether the higher overall mortality after breast cancer diagnosis among women with diabetes is related to a poorer prognosis specific to breast cancer, and there is some indication that premenopausal women develop more aggressive breast cancer subtypes, clinical outcome comprising overall survival and cause-specific survival among breast cancer patients with and without diabetes, also taking into account insulin treatment, should be further evaluated. The data collected in the CARING multi-country study would suit this purpose.

Conclusions

Based on the results presented in this thesis we can conclude that insulin or insulin analogue treatment in patients with diabetes does not increase the risk of breast cancer. There is also no compelling evidence that women with diabetes treated with or without insulin develop different breast cancer subtypes compared to women without diabetes. Although there is some indication that IGF1R and p-MTOR are over-expressed in tumors of insulin users and hormonal receptors are under-expressed in tumors of premenopausal women with diabetes, characteristics that are typically associated with a poor prognosis, based on genes expression analyses these findings were not confirmed. Altogether, it is unlikely that diabetes itself, insulin or insulin analogues strongly affect different pathways involved in breast tumor development. Even though we did not find differences in outcome between women with type 1 and type 2 diabetes, these results are most applicable to women with type 2 diabetes since the number of women with type 1 diabetes was small.
We focused on one type of cancer specifically, since carcinogenic effects of diabetes itself as well as insulin could depend on the tissue in which it is studied and every type of cancer has a different etiology. Therefore, it is hard to extrapolate the findings of this thesis to other types of cancer than breast cancer.

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Chapter 8

Summary & Nederlandse samenvatting

Summary

Diabetes mellitus and breast cancer are two major global health problems with increasing prevalence (**chapter 1**). Meta-analyses reported that women with diabetes have a 20% increased risk of developing breast cancer and a 50% higher risk of death after breast cancer diagnosis as compared to women without diabetes. However, it is unknown whether these associations are due to high blood glucose levels, hyperinsulinemia, shared risk factors such as obesity, side effects of diabetes treatment, and/or due to a different tumor subtype distribution among breast cancer patients with diabetes. An understanding of the link between diabetes, insulin treatment and breast cancer risk as well as subsequent prognosis is important for public health, since a large proportion of the population is affected. If the associations are better understood, it could be determined whether improvements in diabetes care could reduce patients' breast cancer risk and improve prognosis. Therefore, the aims of this thesis were to assess whether diabetes, and specifically insulin treatment, is associated with breast cancer development and breast cancer subtypes, and to investigate potential mechanisms involved (**chapter 1**).

In **chapter 2** we described time-trends and age-specific breast cancer incidence rates (IR) among women with type 2 diabetes in British general practices between 1989-2012, aiming to quantify the double burden of disease and to provide figures for public health policies. A population based-cohort study was conducted in the Clinical Practice Research Datalink. All adult women prescribed anti-hyperglycaemic medication were selected and matched (1:1) on age and clinical practice to a reference cohort without diabetes. We showed that of the 6% of women with prevalent type 2 diabetes in the UK, 2,880 are newly diagnosed with breast cancer each year. This is a high number, but the incidence of breast cancer among women with diabetes remained seemingly stable between 2000-2012, and breast cancer incidence in women with diabetes was similar to incidence in women without diabetes. Therefore, based on this research there is no indication that points towards a need for a different (e.g. intensified) screening approach for breast cancer among women with type 2 diabetes.

In **chapter 3** we reviewed the postulated association between treatment with insulin and/or insulin analogues and breast cancer development, as well as plausible mechanisms involved. We performed a systematic review of *in vitro*, *in vivo* (animal), and epidemiological studies. To study breast cancer risk based on these three types of studies, we made a distinction between tumor initiation and tumor progression as most *in vivo* and *in vitro* studies can only address tumor progression. We concluded that there is no compelling evidence that any of the clinically available insulin analogues (Aspart, Detemir, Glargine, Glulisine or Lispro), nor human insulin, increases breast cancer risk. Overall, the data suggested that insulin treatment is not involved in breast tumor initiation, but might induce breast tumor progression by upregulating mitogenic signaling pathways (e.g. mitogen-activated protein kinase (MAPK)/ phosphatidylinositol 3-kinase (PI3K)).

In **chapter 4, 5,** and **6** we showed results of studies of breast tumors of women with diabetes, treated with or without insulin, compared to breast tumors of women without diabetes. We used data and tumor tissue from primary invasive breast cancer patients diagnosed between 2000-2010, which were randomly selected from an existing nationwide hospital-based cohort in Denmark. Stratified by age at breast cancer diagnosis (≤50 and >50 years), 211 patients with diabetes were frequency-matched on year of birth and age at breast cancer diagnoses to 101 patients without diabetes, with tumor tissue available. The majority (88%) of the women with diabetes had type 2 diabetes mellitus; and 25% of all women with diabetes was treated with insulin. Therefore, the results presented are most applicable to women with type 2 diabetes.

In **chapter 4** we investigated whether women with diabetes develop more aggressive breast cancer subtypes, and whether insulin treatment is related to this. A pathologist stained and scored the tumors for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), antigen Ki67, cytokeratin 5/6 (CK5/6), CK14, and tumor protein p63, and revised morphology and grade. Overall, we found no compelling evidence that women with diabetes develop different clinicopathological subtypes compared to women without diabetes. However, premenopausal women with diabetes tended to have more often PR-negative (OR=2.44 (95%CI: 1.07-5.55)), HER2-negative (OR=2.84 (95%CI: 1.11-7.22)), and basal-like (OR=3.14 (95%CI: 1.03-9.60) tumors than the women without diabetes, with non-significantly increased frequencies of ER-negative (OR=2.48 (95%CI: 0.95-6.45)) and triple negative (OR=2.60 (95%CI: 0.88-7.67) tumors, which are typically associated with poor prognosis. We did neither find strong evidence to support that insulin treatment is associated with clinicopathological breast cancer subtypes; though the poor-prognosis tumors tend to occur more often in premenopausal women with diabetes not using insulin and in type 1 diabetes insulin users.

In **chapter 5** we examined whether proteins within or related to insulin signaling pathways (MAPK/PI3K) are differentially expressed in tumors of women with or without diabetes, treated with or without insulin. We also compared protein expression between users of human insulin and insulin analogues. Tumor samples were successfully stained and scored for phosphorylated-ER (p-ER), epidermal growth factor receptor (EGFR), phosphorylated extracellular signal-regulated kinases (p-ERK1/2), phosphorylated mammalian target of rapamycin (p-mTOR), and insulin growth factor 1 receptor (IGF1R). We found no evidence that the proteins we examined within or related to the PI3K/MAPK pathway were altered in breast tumors of women with pre-existing diabetes. Among women with diabetes, we observed an association between insulin treatment and breast tumors with increased p-mTOR expression (OR=2.35 (95%CI: 1.13-4.88), and in premenopausal women with increased IGF1R expression (OR=5.10 (95%CI: 1.36-19.14)). Among these insulin users, IGF1R was significantly more often expressed in tumors of women treated with insulin analogues compared to women treated with human insulin only (OR=4.94 (95%CI: 1.11-21.92). This observation, if confirmed, might be clinically relevant since the use of IGF1R and mTOR inhibitors are currently investigated in breast cancer clinical trials. We found no strong evidence

for an association between other types of diabetes medication, such as metformin, and any of the proteins that were assessed.

In **chapter 6** we studied the tumor gene expression profiles of 252 of the 312 originally included breast tumors. RNA expression data was analyzed for associations between diabetes/insulin treatment and gene expression, specifically of genes in insulin-related pathways. No significant differences in gene expression of tumors of women with diabetes were found compared to women without diabetes ($p > 0.99$). Expression of insulin-related genes did not differ between tumors of women treated with or without insulin either ($p > 0.98$), nor between women treated with human insulin compared to insulin analogues ($p > 0.46$). Based on this study, it is unlikely that breast tumor etiology is significantly different, or at least differences are very small, in women with and without diabetes and it indicates that exogenous insulin exposure is not an important driver of tumor gene expression.

Concluding, our studies show that insulin or insulin analogue treatment in patients with diabetes is not associated with an increased risk of breast cancer and there is no compelling evidence that women with diabetes treated with or without insulin develop different breast cancer subtypes compared to women without diabetes (**chapter 7**). Altogether, it is also unlikely that diabetes itself, insulin, or insulin analogues, strongly affect insulin-related pathways involved in breast tumor development.

Nederlandse samenvatting

Diabetes Mellitus (suikerziekte) en borstkanker hebben grote invloed op de volksgezondheid en het aantal vrouwen met diabetes en/of borstkanker neemt toe (**hoofdstuk 1**). Publicaties die meerdere studies samenvatten, waarin zowel vrouwen met diabetes type 1 als type 2 werden bestudeerd, laten zien dat vrouwen met diabetes ten opzichte van vrouwen zonder diabetes, een verhoogd risico hebben van 20 procent op het ontwikkelen van borstkanker en van 50 procent op overlijden na diagnose van borstkanker. Het is echter niet bekend welke factoren de oorzaak zijn van het verhoogde risico op borstkanker en de slechtere overleving. Potentiële factoren die mogelijk een rol spelen bij het verhoogde borstkankerrisico onder vrouwen met diabetes zijn: hoge bloedsuikerspiegels, een verhoogd niveau van insuline in het bloed, obesitas en bijwerkingen van diabetesmedicatie (bijvoorbeeld insuline). Daarnaast zouden vrouwen met diabetes mogelijk agressievere borstkankersubtypen kunnen ontwikkelen die geassocieerd zijn met een slechtere overleving. Aangezien een relatief groot gedeelte van de bevolking te maken krijgt met diabetes en/of borstkanker, is het van belang voor de volksgezondheid om het verband tussen diabetes, insuline en het risico op borstkanker, evenals de daaropvolgende prognose, beter te begrijpen. Zo kan worden nagegaan of verbeteringen in diabeteszorg het risico op borstkanker zouden kunnen verminderen en de prognose van borstkanker zouden kunnen verbeteren. Het doel van de onderzoeken in dit proefschrift was om te onderzoeken of diabeten, en in het bijzonder hun behandeling met insuline, samenhangen met het ontstaan en de verdere ontwikkeling van borstkanker en subtypen van borstkanker. Daarnaast is onderzoek gedaan naar mogelijke mechanismen die hieraan ten grondslag liggen.

In **hoofdstuk 2** beschrijven we het nieuw ontstaan (de incidentie) van borstkanker over de jaren 1989 tot 2012 onder vrouwen met type 2 diabetes in verschillende leeftijdsgroepen. Hierbij keken we naar invasieve borstkanker. Dit betekent dat de kankercellen zich verder kunnen verspreiden dan de plek waar ze zijn ontstaan, waardoor vrouwen hier uiteindelijk aan kunnen overlijden. Het doel van dit onderzoek was om het aantal vrouwen met zowel type 2 diabetes als borstkanker over tijd in kaart te brengen. Deze gegevens zouden gebruikt kunnen worden voor het vormen van een verantwoord gezondheidsbeleid (bijvoorbeeld intensievere controles voor borstkanker bij vrouwen met type 2 diabetes). Voor dit onderzoek gebruikten we de gegevens van de 'Clinical Practice Research Datalink; een gezondheidszorgdatabank in het Verenigd Koninkrijk. Hierin selecteerden we een cohort van vrouwen met type 2 diabetes. Deze vrouwen werden op leeftijd en huisartsenpraktijk gekoppeld aan een controlecohort bestaande uit vrouwen zonder diabetes uit dezelfde databank. In het Verenigd Koninkrijk is bij ongeveer 1.92 miljoen vrouwen de diagnose type 2 diabetes gesteld. Op basis van onze data, uitgaande van een soortgelijke leeftijdsverdeling, schatten we dat in het Verenigd Koninkrijk elk jaar 2880 van deze vrouwen met diabetes ook borstkanker ontwikkelen. Het gaat dus om grote aantallen, maar de incidentie van borstkanker bij vrouwen met diabetes bleef betrekkelijk stabiel tussen 2000 en 2012. Daarnaast

was de borstkankerincidentie van vrouwen met diabetes vergelijkbaar met die van vrouwen zonder diabetes. Op basis van dit onderzoek zijn er daarom geen andere, intensievere controles voor borstkanker nodig bij vrouwen met type 2 diabetes.

Om te begrijpen waarom vrouwen met diabetes eventueel een hoger risico op borstkanker hebben, zoals eerder in de literatuur is beschreven, is onderzocht of de behandeling met insuline en insuline-analogen samenhangt met het optreden van borstkanker (**hoofdstuk 3**). Daarnaast hebben we de mechanismen die hieraan ten grondslag kunnen liggen, bestudeerd. Dit hebben we gedaan door alle gepubliceerde *in vitro* (in cellijnen), *in vivo* (in dieren) en epidemiologische studies bij vrouwen systematisch te analyseren. Om op basis van deze drie soorten studies het risico op borstkanker te onderzoeken, hebben we onderscheid gemaakt tussen tumorinitiatie (ontstaan van de tumor) en tumorprogressie (ontwikkeling van de tumorgroei). Wij hebben geconcludeerd dat er geen overtuigend bewijs is dat insuline-analogen of humaaninsuline, die als medicatie worden voorgeschreven aan patiënten met diabetes, het risico op borstkanker verhogen. Op basis van alle beschikbare gegevens lijkt insuline niet betrokken te zijn bij het ontstaan van borstkanker. We vonden echter wel aanwijzingen dat insuline de progressie van borstkanker zou kunnen versnellen door middel van beïnvloeding van bepaalde processen in cellen die de celdeling kunnen versnellen, de zogenaamde mitogene signaaltransductieroutes (e.g. MAPK en PI3K). Dit hebben wij verder onderzocht in de onderzoeken die worden gepresenteerd in **hoofdstuk 5 en 6**.

De resultaten in **hoofdstuk 4, 5 en 6** zijn gebaseerd op een onderzoek waarin we invasieve borsttumoren van vrouwen met diabetes, die behandeld zijn met of zonder insuline, vergeleken met borsttumoren van vrouwen zonder diabetes. We hebben gegevens en tumorweefsel gebruikt van vrouwen bij wie tussen 2000 en 2010 de diagnose borstkanker werd gesteld. Deze borstkankerpatiënten werden in twee groepen willekeurig geselecteerd uit een bestaand Deens nationaal ziekenhuiscohort: vrouwen met een borstkankerdiagnose voor en na het 50ste levensjaar. In totaal werden 211 patiënten met diabetes op basis van geboortejaar en leeftijd bij borstkankerdiagnose gekoppeld aan 101 patiënten zonder diabetes. Van deze patiënten was tumorweefsel, dat bewaard was gebleven, beschikbaar voor dit onderzoek. Vijfentwintig procent van alle vrouwen met diabetes werd behandeld met insuline en het merendeel van vrouwen met diabetes had type 2 diabetes. De resultaten in dit proefschrift zijn daarom voornamelijk van toepassing op vrouwen met type 2 diabetes.

In **hoofdstuk 4** wordt het onderzoek beschreven waarin we hebben onderzocht of vrouwen met diabetes agressievere borstkankersubtypen ontwikkelen en/of er een verband is met insulinebehandeling. Een patholoog kleurde en scoorde de tumoren voor hormoonreceptoren (ER, PR, HER2), een aantal kenmerken voor basale tumoren (CK5/6, CK14 en P63) en een marker voor de snelheid van celdelingen van de tumor (ki67). Ook werden uiterlijke kenmerken van de kankercellen en de graad van agressiviteit van de tumor gescoord. We vonden geen overtuigend bewijs dat vrouwen met diabetes een ander subtype borstkanker ontwikkelen ten

opzichte van vrouwen zonder diabetes. Het bleek echter wel dat vrouwen met diabetes vóór de overgang vaker PR-negatieve, HER2-negatieve en basale tumoren ontwikkelden dan vrouwen zonder diabetes voor de overgang. Ook vonden we aanwijzingen dat vrouwen met diabetes voor de overgang vaker ER-negatieve tumoren ontwikkelden en tumoren waarbij alle drie de hormoonreceptoren niet aanwezig waren, maar deze verschillen waren niet statistisch significant en konden dus ook door toeval worden verklaard. De prognose van deze hormoonnegatieve en basale tumoren is over het algemeen slechter dan van hormoonpositieve tumoren. We vonden ook geen sterk bewijs dat insulinebehandeling samenhangt met specifieke borstkankersubtypen; de tumoren met een slechtere prognose komen echter vaker voor onder vrouwen met diabetes vóór de overgang die geen insuline gebruikten of onder vrouwen met type 1 diabetes.

We onderzochten ook of eiwitten in, of gerelateerd aan, bepaalde processen in cellen die geactiveerd worden door insuline, zogenaamde insulinesignaalroutes (MAPK /PI3K), vaker tot uiting komen in tumoren van vrouwen met en zonder diabetes (**hoofdstuk 5**). Op basis van de studies in hoofdstuk 3 vonden we namelijk aanwijzingen die er op kunnen wijzen dat insuline, via het afgeven van deze signalen aan cellen, de progressie van borstkanker zou kunnen stimuleren. In tumoren van vrouwen met diabetes vergeleken we het tot uiting komen van eiwitten tussen insuline- en niet-insulinegebruikers en maakten we onderscheid tussen gebruikers van humaaninsuline en insuline-analogen. De tumorweefsels werden gekleurd en gescoord voor hormoon- en groeifactoren in, of gerelateerd aan, insulinesignaalroutes (p-ER, EGFR, p-ERK1/2, p-mTOR en IGF1R). Wij vonden geen verschil in het tot uiting komen van bovengenoemde eiwitten tussen tumoren van vrouwen met en zonder diabetes. Vrouwen met diabetes die behandeld werden met insuline, hadden borsttumoren waarbij de eiwitten p-mTOR en IGF1R vaker tot uiting kwamen. Onder de insulinegebruikers kwam IGF1R vaker tot uiting in tumoren van vrouwen die behandeld waren met insuline-analogen ten opzichte van vrouwen die alleen behandeld werden met humaaninsuline. Als toekomstige studies bevestigen dat insuline(analoog)gebruikers vaker IGFR- en p-mTOR-positieve tumoren ontwikkelen, zou dit klinisch relevant kunnen zijn. Het gebruik van IGF1R- en mTOR-remmers voor de behandeling van borstkanker wordt momenteel namelijk onderzocht in klinische studies. Wij vonden geen bewijs dat andere soorten diabetesmedicatie, zoals metformine, invloed hadden op het tot uiting komen van eiwitten in of gerelateerd aan insulinesignaalroutes.

In **hoofdstuk 6** hebben we de mate van tot uiting komen van genen (genexpressie) in de bovengenoemde tumoren beschreven. Dit deden we door middel van het aflezen van alle genen, op basis van RNA dat geïsoleerd was uit de tumor (RNA-sequencing). We onderzochten de verbanden tussen genexpressie en diabetes met en zonder insulinebehandeling, in het bijzonder voor insulinegerelateerde genen. We vonden geen verschillen in tumorgenexpressie tussen vrouwen met diabetes en vrouwen zonder diabetes. Ook vonden we geen verschil in expressie van insulinegerelateerde genen tussen tumoren van vrouwen die met en zonder insuline werden behandeld, noch tussen humaaninsuline- en insuline-analooggebruikers. Op basis van

dit onderzoek is het onwaarschijnlijk dat de processen die betrokken zijn bij de ontwikkeling van borstkanker sterk verschillen tussen vrouwen van vergelijkbare leeftijd met en zonder diabetes. Mogelijk zouden er wel subtiele verschillen op te sporen zijn geweest als we deze studie op ingevroren tumoren hadden uitgevoerd, maar deze waren in zulke aantallen niet voorradig.
De belangrijkste conclusie die we trekken in dit proefschrift is dat behandeling met humaaninsuline of insuline-analogen bij patiënten met diabetes het risico op borstkanker niet verhogen (**hoofdstuk 7**). Daarnaast is er geen sterk bewijs dat het subtype borstkanker dat een vrouw ontwikkelt, gerelateerd is aan het wel of niet hebben van diabetes en/of behandeling met insuline. Gezien onze resultaten is het onwaarschijnlijk dat diabetes zelf, gebruik van humaaninsuline of insuline-analogen een sterke invloed hebben op (insulinegerelateerde) signaalroutes die betrokken zijn bij de ontwikkeling van humane borsttumoren.

8

Appendices

Publications

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About the author

Publications

H.K. Bronsveld*, B. ter Braak*, O. Karlstad, P. Vestergaard, J. Starup-Linde, M.T. Bazelier, M.L. De Bruin, A. de Boer, C.L. Siezen, B. van de Water, J.W. van der Laan, M.K. Schmidt. Treatment with insulin (analogues) and breast cancer risk in diabetics; a systematic review and meta-analysis of in vitro, animal and human evidence. *Breast Cancer Research 2015, 17(1):100*
*Authors contributed equally

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H.K. Bronsveld, M.L. De Bruin, J. Wesseling, J. Sanders, I. Hofland, V. Jensen, M.T. Bazelier, B. ter Braak, A. de Boer, P. Vestergaard, M.K. Schmidt. The association of diabetes mellitus and insulin treatment with expression of insulin-related proteins in breast tumours. *Submitted.*

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Not in this thesis:
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About the author

Heleen Katrien Bronsveld was born on March 1st 1986 in Sint-Niklaas, Belgium. In 2004 she completed secondary school at the "Twickel college" in Hengelo. Thereafter she moved to Wageningen, the Netherlands, to study Nutrition and Health. During her Bachelor of Science degree she was a visiting student at Cambridge University, United Kingdom. She continued to pursue a Master of Science in Nutritional and Public Health Epidemiology at Wageningen University. For her Master thesis she worked at the Channing Laboratory at Harvard Medical School in Boston. Under supervision of dr. Rulla Tamimi and prof. dr. Ellen Kampman, she investigated whether dietary flavonoid intake influenced breast density and subsequent risk of breast cancer among women in the Nurses' Health Study. After she graduated cum laude in 2010, she was employed as product development scientist/nutritionist at Mars Inc. for two years.

In September 2012 she started her PhD at the Division of Molecular Pathology and the Department of Psychosocial Research and Epidemiology, the Netherlands Cancer Institute – Antoni van Leeuwenhoek, in cooperation with the division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University. She also regularly travelled to Denmark for a study visit to Aarhus University Hospital. The results obtained in this period are described in this thesis. She presented the results on several national and international conferences.

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