Chapter 2

Insulin treatment and breast cancer risk; a review of in vitro, animal and human evidence

Highlights

- The number of animal studies on the carcinogenic potential of insulin analogues is low
- Epidemiological studies on this topic were underpowered
- Both epidemiological and in vitro studies on this topic suffered from methodological limitations
- There is no compelling evidence that any clinically available insulin analogue increases breast cancer

This chapter has been submitted as:

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◀ IN THE PICTURE

Western blot analysis. This technique is widely used to quantify specific protein levels in a sample (a dark band indicates more protein). It became one of my favourite read-outs and plenty of WB overviews are presented in this thesis (during my PhD over 750 individual blots have been performed). In my opinion this technique is undervalued. In comparison to "State of the art" techniques like immunofluorescence, the protein levels are better quantifiable, results better reproducible, and easier to interpret.

◀ IN BEELD

Western blot analyse. Deze techniek wordt wereldwijd gebruikt om eiwit niveaus te bepalen in een monster (een donkere band betekent meer eiwit). Het is een van mijn favoriete technieken en er staan veel WB overzichten in deze thesis (meer dan 750 individuele blots zijn uitgevoerd gedurende mijn PhD). Ik vind dat dat deze techniek door onderzoekers wordt ondergewaardeerd. In vergelijking met de "hippe" technieken, zoals immunofluorescentie, zijn de eiwitniveaus beter kwantificeerbaar en de resultaten gemakkelijker te duiden.

Abstract

The association between insulin and insulin analogue treatment and breast cancer development, and plausible mechanisms, was investigated. 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 and a complete 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. In total 16 in vitro, 5 animal, 2 in vivo human and 29 epidemiological papers were included in this review. Insulin AspB10 showed mitogenic properties in in vitro and 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 increased breast cancer risk (HR 1.04; 95% CI 0.91, 1.17; p=0.49). 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. There is no compelling evidence that any clinically available insulin analogue (Aspart, Determir, Glargine, Glulisine or Lispro) 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.

Keywords: Breast cancer, insulin analogues, diabetes mellitus, systematic review, meta-analyses, epidemiology, animal studies, in vitro, glargine

Introduction

Breast cancer is the most prevalent cancer in women with 1.67 million new cancer cases diagnosed in 2012 worldwide [7]. There is evidence that diabetes mellitus (DM) is associated with breast cancer [8-13]. 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 such as insulin. Insulin can act as a growth factor, and it is biologically plausible that high levels of endogenous insulin or exposure to exogenous insulin could stimulate neoplastic growth [14, 15].

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 overall [16-20]. 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 [21].

Previous reviews that focussed on in vitro studies were consistent on the note that glargine has, in contrast to other commercially available analogues, an increased binding affinity towards the Insulin-like growth factor 1 receptor (IGF1R). Most studies concluded that glargine may have an increased mitogenic potential in particular cell lines at supra-physiological concentrations [22, 23]. Extrapolation of these results to the human in vivo situation 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.

Moreover, no studies have reviewed the limited number of animal studies on insulin analogues and cancer, so far. In addition, meta-analyses of epidemiological studies have been inconsistent. One meta-analysis reported an increased relative risk of any cancer among insulin users compared to non-insulin treated diabetics of 1.39 (95% Confidence Interval (CI) 1.14, 1.70) [24], while another reported a pooled estimate of 1.04 (95% CI 0.75, 1.45) [25]. Insulin use was not associated with an increased risk of breast cancer [24-26]. However, two [25, 26] out of four meta-analyses [25-28] 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. Furthermore, we deducted from the literature review what is currently known on signalling pathways involved in insulin induced tumorigenesis. We included all widely prescribed insulin analogues and insulin AspB10 and included in vitro, animal, in vivo human and epidemiological studies.

Methods

This systematic review is registered at PROSPERO [29] with the registration number: CRD42012002477 and was developed according to the PRISMA guidelines [30], and supplemented by guidance from the Cochrane Collaboration handbook [31].

Data sources and searches

A search of three online databases, 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 displayed in the electronic supplementary material (ESM) 1.

Study selection

Eligible studies had to describe effect measures of exogenous insulin 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 3 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 Fig. 1.

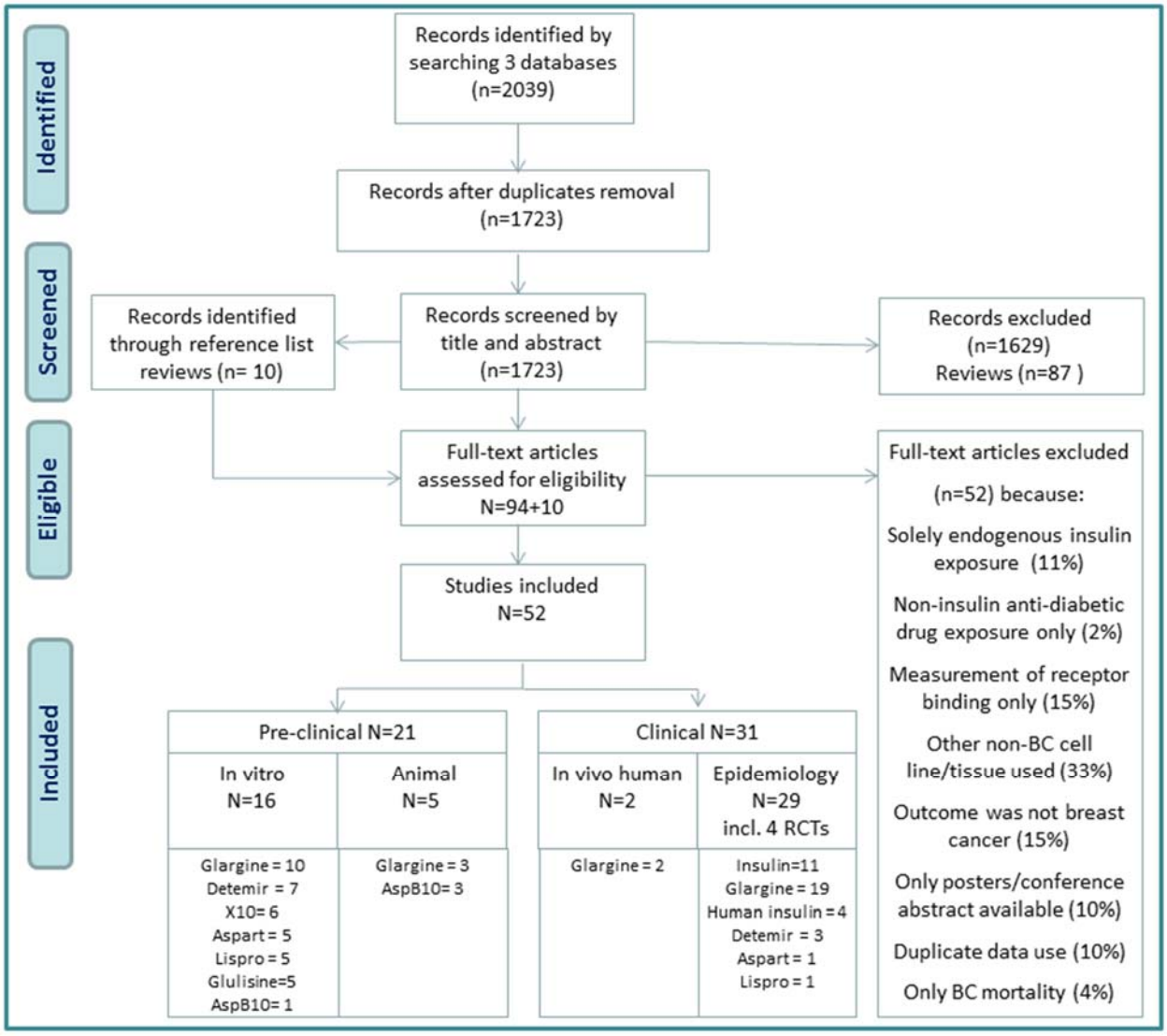

Fig. 1 Flow chart of study identification and study selection process.

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 8 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) insulin use versus no insulin use (drug exposure undefined); 2) insulin use 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.

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

Author, Year	Cell-line	IR : IGF1R (ratio)	Method	Starvation	Stimulation time	Refreshment of medium	Type of stimulation medium	Presence phenol red	Analogue tested	Concentrations tested nM	Mitogenic response	Sig.	PI3K path-way*	MAPK path-way*
Milazzo et al, 1997	MCF7[A] MCF10[B]	1 : 4 1 : 0.8	[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		
			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	MCF7[A] MCF10[B]	- -	[3H]Thymidine incorporation	48h[A] 24h[B]	20 hrs stim 4 hrs measure	Yes	DME/F12 SFM	No	Glargine	10, 50, 100	↓[A]	No		
			MTT	No	4 days	Yes, every two days	DME/F12 SFM	No	Glargine	1, 5, 10, 25	↑[A,B]	No		
Liefvendahl et al, 2008	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	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	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	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			Yes	↓[AB]	↓[AB]
Weinstein et al, 2010	MCF7	-	Cell counting	No	72 hrs	Yes every day	DMEM/SFM		Glargine Detemir	100	↑ ↑	No No		
Oleksiewicz et al, 2011	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	MCF7[A]	-	MTT	24 hours	2 days	Yes, every two days	RPMI + 0.5 % CS-FBS	No	Glargine	20-200	↑[A]	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	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	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	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	MCF7[A] MDA-MB-231[B]	- -	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]	
			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] - -			

<To be continued on next page>

Table 1. (part 2) Overview of *in vitro* studies in breast cancer cell lines on the mitogenic potential of insulin analogues.

Author, Year	Cell-line	IR : IGF1R (ratio)	Method	Starvation	Stimulation time	Refreshment of medium	Type of stimulation medium	Presence phenol red	Analogue tested	Concentrations tested nM	Mitogenic response	Sig.	PI3K path-way*	MAPK path-way*
Gallagher et al, 2013	MET1 MVT1	- -	WB	1 hr	10 min	No	DMEM + 0.1% BSA		X10	10	↑	Yes		
Ter Braak et al, 2014	MCF7 IGF1R[A] MCF7 IRA[B] MCF7 IRB[C]	1:25 1:0.02 1:0.07	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]	
			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	MCF7[A] MDA-MB-157[B] MDA-MB-468[C] T47D[D]	1:6 1:2 1:0.2 1:8	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]		
			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 on the correlation of insulin analogues and breast cancer

Author, Year	Model	# 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 (re-evaluation in 2012)	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, 2013	orthotopic mammary tumour wt and hyperinsulinaemic MKR mice	3-4	Mammary gland Lung metastasis	0-25 days	AspB10	12.5 IU/kg 2x/day	Tumour volume measurement		↑	Yes	
							Counting Lung metastasis		↑	No	
							WB receptor activation	p-IR/ p-IGF1R p-Akt p-Erk	↑ ↑ -	Yes Yes	
Tennagels et al, 2013	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	p53[R270H/+]WAP Cre FVB mice	40	Mammary gland tumors	Chronic exposure till MG tumor development	Glargine AspB10	12.5-15 IU/kg 150-200 IU/kg	Tumour latency time		↑ ↑	No Yes	majority aggressive EMT no correlation pathology and treatment
							WB protein expression profiling	IR, IGF1R, Erk, p-Erk, Akt, p-Akt, EGFR, ER, E-cad, N-cad, Her2	↑ ↑	Yes Yes	

Table 3. Relative risk estimations for breast cancer among different insulin treatment groups and the evaluation of bias and power of the epidemiological 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
Insulin – no insulin: Hazard Ratio								
Carstensen et al, 2012	Insulin users	No insulin users	248/102,500	2,118/627,100	0.96	0.84-1.09	Moderate	Adequate
Ferrara et al, 2011	Insulin users	No insulin users	NR	NR	1.0	0.9-1.2	Moderate	Adequate
Gu et al, 2013	Insulin users	No insulin users	4/6,188*	14/10,435*	0.33	0.10-1.13	Moderate	Too low
Neumann et al, 2012	Insulin users	No insulin users	NR/NR*	NR/NR*	**0.86**	**0.81-0.91**	High	Adequate
Onitilo et al, 2014	Insulin users	No insulin users	NR/NR*	NR/NR*	0.84	0.58-1.23	High	Too low
Insulin – no insulin: Odds Ratio								
Bodmer et al, 2010a	Insulin users	No insulin users	43/131	262/1,022	NE	NE	High	Too low
Cleveland et al, 2012	Insulin users	No insulin users	20/16	50/49	1.15	0.40-3.40	High	Too low
Insulin - NIAD: Hazard Ratio								
Currie et al, 2009a	Insulin users	Metformin only	NR/12,640*	NR/34,847*	1.07	0.79-1.44	Moderate	Too low
Redaniel et al, 2012a	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	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******	Pioglitzone users, not using insulin	Insulin users, not using pioglitazone	181/29,721	113/13,680	0.85	0.67-1.08	High	Low
Insulin - NIAD: Odds Ratio								
Hsieh et al, 2012	Insulin only users	Metformin only users	5/NR	19/NR	1.63	0.60-4.40	High	Too low
Koro et al, 2007a	Insulin and NIAD users	TZD users	13/52	83/449	0.71	0.36-1.37	High	Too low
Koro et al, 2007b	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*****	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*****	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	Glargine users	NPH users	3/2,144	5/2,096	0.90	0.64-1.26	Low	Too low
Chang et al, 2011*****	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	Glargine plus non-glargine insulin users	Non-glargine insulin users	0/NR	29/9,667*	NE	NE	High	Too low
Colhoun et al, 2009b*****	Glargine only users	Non-glargine insulin users	6/1,200*	29/9,667*	1.47	0.59-3.64	High	Too low
Currie et al, 2009b*****	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*****	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**** *	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	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	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*****	Glargine users	NPH insulin users	NR	NR	0.93	0.68-1.27	High	Too low
Lind et al, 2012a*****	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	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*****	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*****	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 ****	Glargine users	NPH users	103/26,277	19/5,885	1.07	0.65-1.75	Moderate	Too low
Suissa et al, 2011a*****	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	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	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	Glargine users	Non-glargine users	78/287	697/2,763*	1.04	0.76-1.44	Low	Borderline
Grimaldi-Bensouda et al, 2013b	Glargine users	Non-glargine insulin users	74/203	70/207	0.96	0.61-1.53	Low	Too low
Grimaldi-Bensouda et al, 2013c	Glargine users	Human insulin users	NR	NR	1.29	0.78-2.13	Low	NE
Grimaldi-Bensouda et al, 2013d	Glargine users	Aspart users	NR	NR	1.10	0.64-1.89	Low	NE
Grimaldi-Bensouda et al, 2013e	Glargine users	Lispro users	NR	NR	0.85	0.48-1.50	Low	NE
Mannucci et al, 2010a	Glargine users	Non-glargine insulin users	NR	NR	NE	NE	High	Too low
Determir – no determir: Hazard Ratio								
Fagot et al, 2013b	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	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	Determir users	NPH users	1/2,252	0/1,420	NE	NE	Low	Too low
Dejgaard et al, 2009b	Determir users	Glargine users	1/917	3/628	NR	NR	Low	Too low
Aspart – no aspart: Odds Ratio								
Grimaldi-Bensouda et al, 2013f	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	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	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
Ruiter et al, 2012b	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	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.

Results

A search in MEDLINE at PubMed, EMBASE, and ISI Web of Science identified 1723 unique records (Fig. 1). After the eligibility assessment, 52 studies on exogenous insulin 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 [32-47]. Protein expression of hormone receptors and some downstream signalling proteins for each cell line are provided in ESM Table 1 and Fig. 2. In Table 2 an overview is presented of all relevant animal studies [48-52]. Descriptions and characteristics of the epidemiological studies are presented in ESM Table 2-3c [18, 19, 53-79]. 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 Fig. 1. Results of the meta-analysis on glargine can be found in Fig. 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.

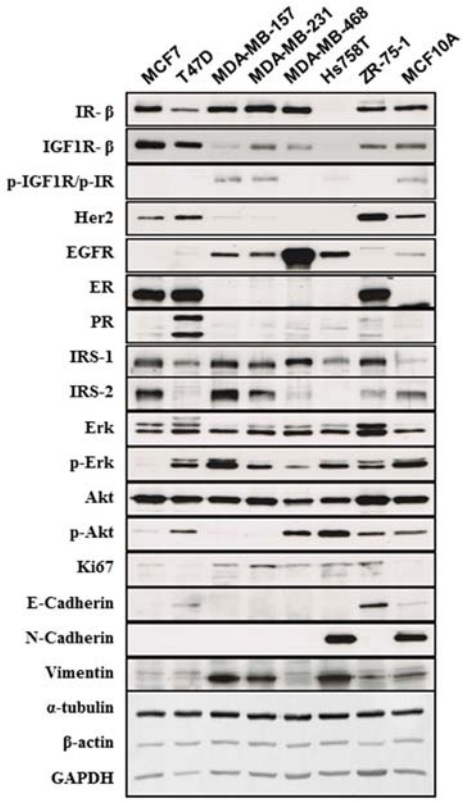

Fig. 2 Protein expression profiling of eight commonly used human breast cell lines. Receptor levels and signalling molecules downstream of the INSR/IGF1R signalling pathway have been quantified. Furthermore some breast cancer subtype markers have been used to further characterize these cell lines that are commonly used in the research articles discussed in this review.

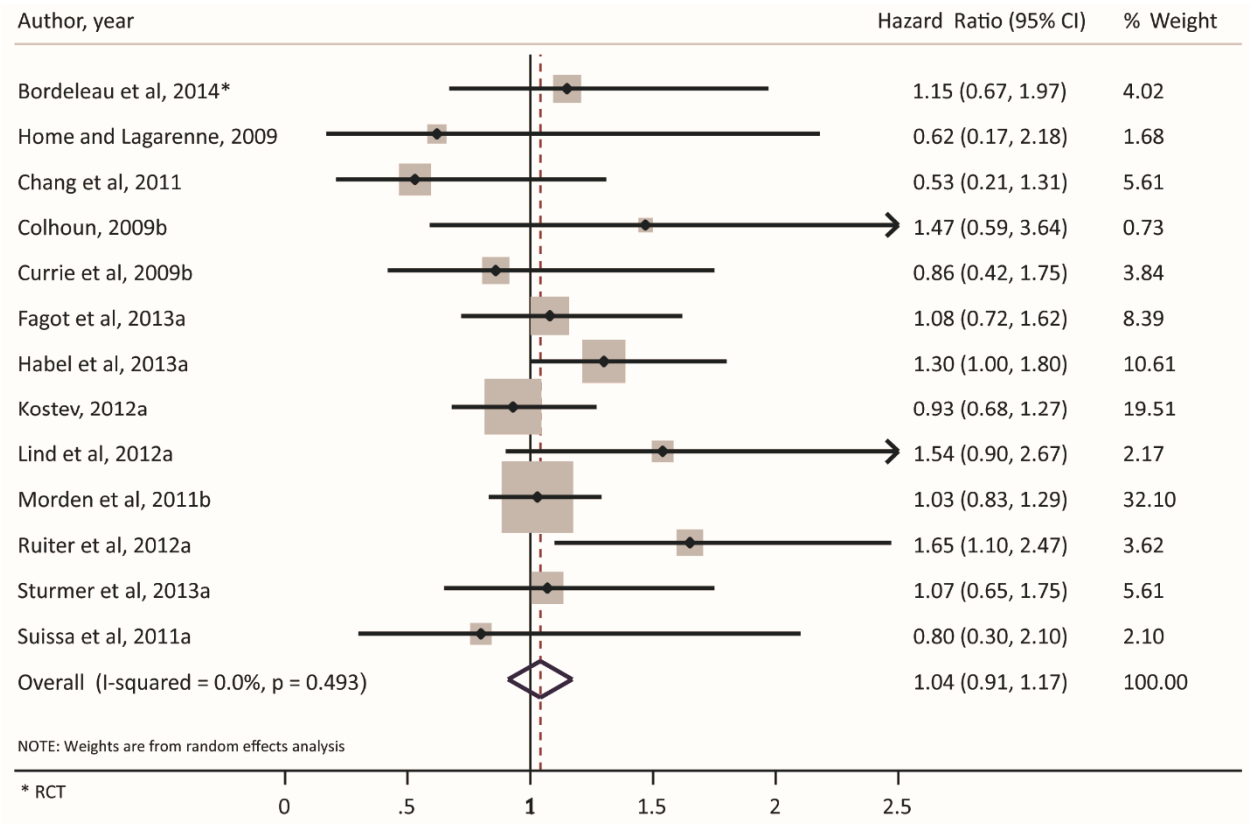

Fig. 3 Forest plot reported hazard ratios for risk of breast cancer among insulin glargine users.

Insulin AspB10

The increased carcinogenic effect of insulin AspB10 was already discovered in 1992 [80]. 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 [33, 34, 38, 39, 41, 46] (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 [38]. 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 [32]. 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 [34]. 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 [41]. 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 [39].

In animal studies, AspB10 was found to have a dose-dependent increased carcinogenic potential[80] (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 [32]. 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 up regulated after AspB10 and IGF1 treatment [52]. A different study focussed on the short term mitogenic effects of AspB10 and found a significant stronger receptor activation in the mammary glands of Sprague-Dawley rats one hour after AspB10 injections compared to human insulin treatment [51].

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 glargine (M1/M2)

Seven of ten in vitro studies found an increased proliferative potential of glargine in comparison with human insulin [34, 37, 40, 41, 43, 46, 47] (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 [44, 45]. One study is difficult to interpret, since IGF1 did not show an increased mitogenic potential either [36]. Glargine has, similar to insulin AspB10, an increased binding affinity towards IGF1R [81]. 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 up regulated after glargine stimulation compared to human insulin stimulation [40, 43, 45, 46]. Two studies also found the MAPK signalling cascade to be up regulated [40, 43]. 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 [82, 83]. These metabolites possess low mitogenic signalling [40, 46].

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 [49, 50] (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 [52]. Glargine injections induced no increased receptor activation response in the mammary glands of Sprague-Dawley rats [51].

Three Randomized Clinical Trials (RCT) that investigated breast cancer risk among glargine users compared to non-glargine users [54, 64, 75] did not show significant differences (Table 3). Most case-control and cohort studies showed a non-significant increased risk. Only two observational studies [69, 76] 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 [69] and human insulin only users [76]. 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) (Fig. 3 and Table 3), showing no evidence for an association between insulin glargine treatment and an increased incidence of breast cancer.

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 [34, 40, 43, 46], but in a number of in vitro studies a similar or even an increased proliferative behaviour was observed [37, 41, 47] (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 (fetal 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 [84]. For the same reason it is not surprising that no chronic animal studies have been conducted with insulin detemir. Only 3 epidemiological studies have been performed, one RCT [58] and two cohort studies [59, 67]; 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 [37, 40, 42, 46] (Table 1). Only one in vitro study suggested a small non-significant proliferative increase of aspart compared to human insulin [43]. Another in vitro study found the mitogenic potential of glulisine to be significantly lower than human insulin [42]. 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) [41]. We previously found that the PI3K signalling cascade is significantly more up regulated after lispro treatment than human insulin stimulation only in the IGF1R over expressing MCF7 cell line [46]. 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 [61] (Table 3).

Insulin users versus non-insulin 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). 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 [72]. However, we judge this study is likely to be biased.

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 [53, 55, 74] and insulin glargine [61, 63, 68, 77, 78] (ESM Table 4). However, a non-significant increased risk was found after more than 5 years of any insulin treatment (HR 2.25; 95% CI 0.72, 6.99) [74]. Among the glargine users, the study with the longest follow-up comparing exposure of 4-7 years versus <4 years did not observe an increased breast cancer risk [61]. Another study revealed that the risk of breast cancer increased in the first 3 years after start of insulin glargine use. After 3 years risk of breast cancer remained at the same level [68]. Results

of glargine dose on the occurrence of breast cancer [59, 61, 68, 70, 71, 76] showed inconsistent results (ESM Table 4). Some studies found significant increased relative risks with increasing dose [68, 71, 76], while others did not [59, 61, 70, 71]; this seems partly dependent on the exposure definition. Only one of the studies investigating glargine dose used cumulative dose [59]. 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 [83].

Discussion

Based on the current epidemiological and animal data there is no compelling evidence that any clinically available insulin analogue increases breast cancer risk. However, animal data was limited while the epidemiological studies were underpowered and suffered from methodological limitations. 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.

Limitations of the studies and interpretation of the findings

In vitro studies

The main reason for contradictory in vitro findings can be explained by differences in study design. The responsiveness to growth factors, like insulin and insulin analogues, is to a large extent dependent on the cell line that is used in the assay. Based on the cell characterization (ESM Table 1), there is a striking variation in receptor expression of the human cell lines used. The MDA-MB cell lines are characterized by high levels of the INSR but low levels of the IGF1R compared to MCF7. Therefore, studies that used both cell lines could detect an increased mitogenic potential of IGF1 and glargine due to enhanced IGF1R signalling only in the MCF7 cell line, but not in the MDA-MB-231, as expected [40]. 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. However, the authors concluded that the INSR/IGF1R status was not the only explanatory factor [41]. Therefore, expression of downstream signalling molecules has also been determined (Fig 2). This gives insight into the lack of responsiveness of MCF10A when exposed

to glargine [37, 43, 44], since this cell line has low expression of IRS1, the first downstream target of the INSR/IGF1R.

The majority of the mitogenicity studies used the MCF7 cell line [35-40, 42-47]. 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 [33, 37, 40, 45], 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 [44, 45] did not include a positive control while others lack a negative control [35], thereby making it impossible to put the results in perspective. Furthermore, one study did include a positive control (IGF1) [36], 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 [85]. 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 [49-51]. 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 [52]. In another study a high mortality was observed, probably due to hypoglycaemia, therefore the dose was lowered in a later phase of this study [51]. Surprisingly, other studies that used similar doses did not observe hypoglycaemia [49, 50, 52]. 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 [76], while others compared glargine users with non-glargine insulin users [78]. In this case, the comparator is a mix of several exposures. Some studies examined several definitions for the exposure of interest and this resulted in slightly different effect estimates [69, 71]. Besides that, these categories do not specify whether these insulin users were using different NIADS at the same time.

Inclusion criteria differed largely among studies. For example, some studies included patients with only 1 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 5 studies [54, 58, 61, 74, 75], but in these studies power was not adequate (ESM Table 5). Of these studies, only two studies considered breast cancer as a main outcome [61, 74]. 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 [69, 72] or even so had lack of power [69, 76]. So far there is not a single properly 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 6 years [54]), insufficient power, or cancer incidence as a secondary outcome [75, 86].

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.

Tumour promotion is related to tumour subtype and survival. Compared to patients without DM, breast cancer in patients with DM is often diagnosed at an advanced stage [87-92]. However, only two studies reported information on breast cancer subtypes after insulin treatment. One in vivo study reported more PR- (38% vs. 26%) and less HER2+ (0% vs. 6%) tumours among glargine users compared to patients using other types of insulins [93]. One epidemiological study provided the occurrence of breast cancer subtypes among glargine users (HER2+: 8.1%, triple negative: 14.8%, luminal: 9.0%) [61]. It has been shown that overall mortality after breast cancer diagnosis is 30 to 40% higher in diabetic women compared to their non-diabetic counterparts [90, 94-101]. Whether this increased mortality is caused by death due to breast cancer or death by comorbidities related to DM is not clear. One study found that the increased risk of dying in DM with breast cancer is comparable to the general increased risk of dying as a diabetic patient [102]. Studies that investigated the association between breast cancer-specific mortality and diabetes show inconsistent results [87, 90, 91, 94, 103]. Among patients with type 2 DM, insulin treatment was associated with a worse cancer outcome and increased all-cause mortality compared to metformin treatment [90, 104]. Only one study investigated the effect of cumulative dose and duration of insulin treatment on breast cancer specific survival, and found a lower breast cancer mortality [105].

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 [106].

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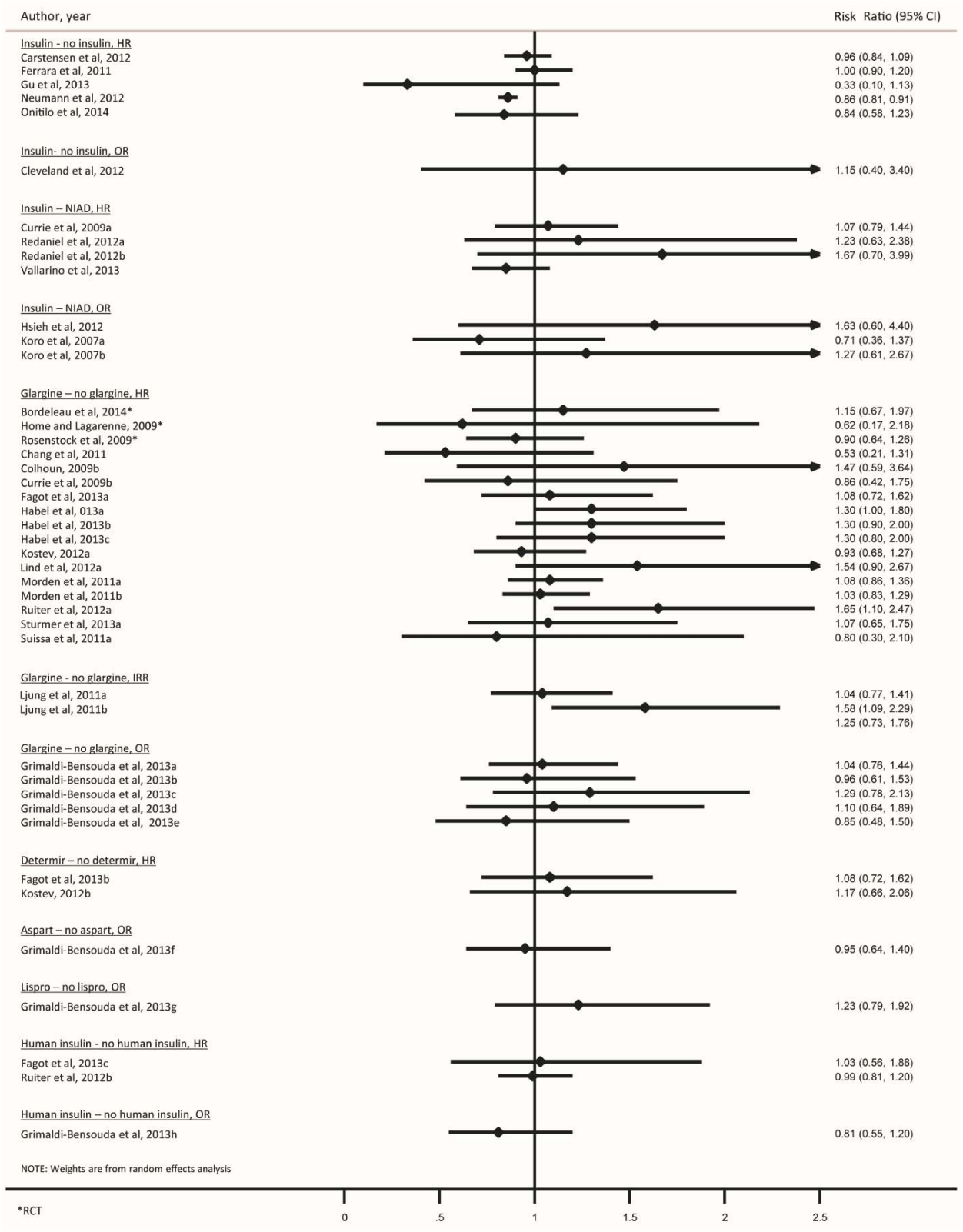
Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement

MKS, JWVDL, CLES, BVDW and ADB conceived this study. HB and BTB extracted and analysed the data. BTB performed the protein quantification experiments on the cell lines. HB and ØK evaluated study quality of the epidemiological studies. HB, BTB, MKS, and CLES interpreted the data and wrote the manuscript. All authors critically read and approved the final version of the manuscript.

Supplemental Figure



ESM Fig. 1 Forest plot of breast cancer risk among insulin (analogues) users stratified by treatment group and type of effect estimate. Different exposure comparisons within one study are indicated by a, b, c. The exposure comparison can be found in Table 3 and ESM Table 2