Carcinogenicity of insulin analogues

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Chapter 1

Aim and scope of thesis

Highlights

- Insulin analogues are widely used by diabetic patients to control blood glucose levels
- The use of some of these compounds is correlated with an increased cancer risk
- The medicine administration agencies propose new carcinogenic risk assessment strategies for insulin analogues

This chapter is adapted from:

M. Dempster, C.L.E. Siezen, B. ter Braak, W. van den Brink, A. Emerenciana, F. Bellanti, R.G. Duijnhoven, M. Kwa, J.W. van der Laan

Carcinogenicity of Biophamaceuticals

In press, March 2015, "Genotoxicity and Carcinogenicity Testing of Pharmaceuticals", Springer Press

And

B. ter Braak

Anti-diabetic insulin analogue drugs and breast cancer development

Februari 2015, Guest Blog on biomedcentral.com

◀ IN THE PICTURE

Insulin analogue pens and refills. Diabetic patients will certainly recognise these user friendly injection pens. Using the rotating cap the patient can adjust the required dose. The needle can be mounted on top of the pen. Typically, the thigh or belly is used as suitable injection site. All analogue flasks (as well as those of regular insulin, IGF1 and glargine metabolites) in the picture have been used in this research. Recently it appeared that IGF1 was misused to enhance sport performances by bodybuilders and cyclist.

◀ IN BEELD

Insuline analogen pennen met navulsysteem. Diabetes patiënten zullen deze gebruiksvriendelijke pennen zeker herkennen. Met de roterende dop kan de dosis ingesteld worden. De naald wordt op de voorkant geschroefd. Normaal gesproken wordt de dij of buik gebruikt als injectieplaats. Alle analogen flesjes (evenals die van normaal insuline, IGF1 en de metabolieten van glargine) op de foto zijn gebruikt in dit onderzoek. Recent is gebleken dat IGF1 is misbruikt door bodybuilders en wielrenners om hun sportprestaties te verbeteren.

Every year hundreds of new chemical entities are produced intended for the human pharmaceutical market. Many of these compounds will never reach the market or will eventually be redrawn from it because of serious side effects. The process to get safe medicines on the market is regulated by national authorities of each individual member state in Europe, and a centralized organization called the European Medicines Agency (EMA).

The demands for the development of a medicinal product are laid out in numerous guidelines, drawn up by the regulatory authorities. A number of these guidelines is aimed at providing guidance on the non-clinical development and risk assessment of new products, mainly of the small chemical type. However, because of their unique biological and physiochemical characteristics, a specific guideline was written for biotechnology-derived compounds which is based on a scientific case-by-case approach [1] [2].

Although biopharmaceuticals are not genotoxic and therefore are not expected to be 'complete carcinogens', chronic administration could potentially lead to tumor promotion or progression of specific neoplasm(s) based on their expected pharmacologic activity [3]. In several scenarios evaluation of the carcinogenic potential should be considered for non-genotoxic biopharmaceuticals:

1. In case different biological effects are observed between the recombinant product and the endogenous protein.
2. When there are structural differences between the recombinant product and natural product.
3. If the recombinant products are administered at pharmacologic doses greater than expected endogenous levels.

But emphasizing a 2-year rodent study (rat or mouse), and thus referring to rodents as a golden standard is neglecting the high number of false positives in these species as compared to humans. Half of all long- term used pharmacotherapies induces rodent cancer, due to the high sensitivity of rodents versus humans, human irrelevant age-related tumors arise in these animals [4] [5]. The purpose of the S6 guideline with the recent addendum was to offer alternative strategies rather than to default to the rodent bioassay to provide an appropriate carcinogenic risk assessment [2]. Alternative strategies include in vitro approaches and if necessary a more relevant rodent in vivo model, besides a review of available literature data, information from similar targets or class effects, and clinical data.

The molecules discussed in this thesis are the insulin analogues, these molecules are very similar to regular insulin but have improved pharmaco-kinetic and -dynamic parameters. Insulin analogues are used by diabetic patients to regulate their blood glucose levels. Long- and short-acting insulin analogues have been developed so that plasma levels can be tuned accurately during the day reflecting the physiological activity of endogenous insulin without much fluctuation after physical activity or food intake.

Insulin analogues (as well as regular insulin) are growth factors and have besides the intended metabolic effects also an intrinsic mitogenic behavior. While the pharmacological action of insulin is mediated through the insulin receptor, the mitogenic potential of insulin and insulin analogues are mainly related to their affinity for and downstream effect via the insulin like growth factor-1 receptor (IGF1R) (Figure 1). The natural ligand of the IGF1R, IGF1, is structurally very similar to insulin which results in cross-reactivity of the different ligands for the above mentioned receptors. An increased binding affinity towards the IGF1R compared to regular insulin could be the result, which is indeed the case for some insulin analogues. Therefore a major concern with respect to safety aspects for insulin analogues is a disproportional increased mitogenic activity.

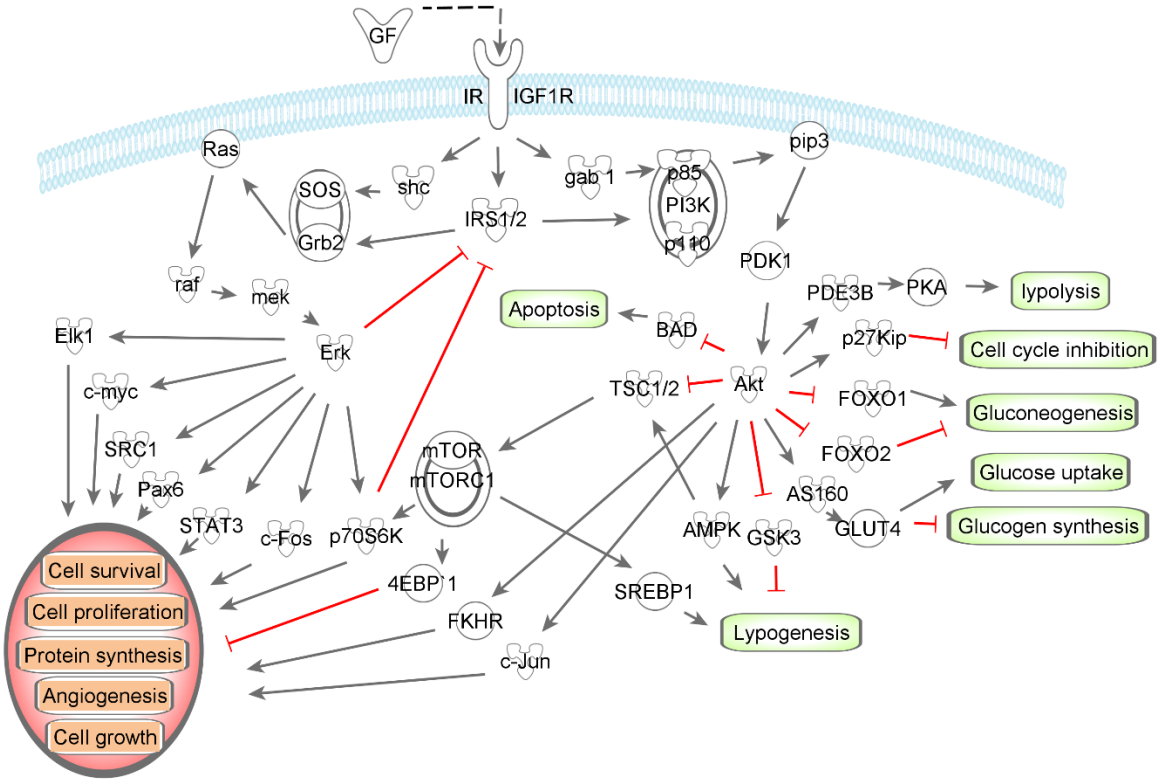


Figure 1. The insulin receptor (IR) and insulin like growth factor-1 receptor (IGF1R) signalling pathway. Activation of these receptors by a growth factor (e.g. insulin or insulin analogues) triggers its auto-phosphorylation. Several substrates (e.g. shc and gab1) can bind the membrane bound receptor and get phosphorylated, which in turn will activate two distinct signalling cascades, the PI3K/AKT and MAPK/ERK. The PI3K is thought to play a major role in metabolism (e.g. glucose uptake, glucogen synthesis), whereas the MAPK leads to the more mitogenic effects (e.g. cell proliferation and survival). But as is clear from the pathway many cross links can be made between these different cascades, making the IR/IGF1R signalling pathway highly complex.

In 2002, the EMA proposed testing strategies for insulin analogues specifically in a guidance document [6], in which it was stated that the preclinical safety evaluation of these compounds should focus on the mechanisms of action of the expected carcinogenic effect. Besides receptor kinetics and binding affinity, characterization of the different intracellular pathways is needed, not only for the IGF1R both also for the different isoforms of the insulin receptor. There is

evidence that these receptors might react differently in neoplastic tissue compared to normal tissue, therefore in vitro studies should be performed in both cancer cell lines and primary cells to make a comparison of insulin analogue induced pathway activation. The use of adapted animal models is encouraged to increase the human clinical relevance of these chronic rodent experiments. Furthermore, the importance of including the right reference compounds was highlighted. Native insulin, IGF1 and AspB10 (an insulin analogue with a known increased mitogenic activity) should be included in the mitogenic assays to put the obtained results in perspective.

In this thesis we have used the recommendations from the EMA as a guideline for the carcinogenic risk assessment of all commercial available insulin analogues.

In **chapter 2**, an introduction is presented that included a literature search of all available experimental data on this topic. For this systematic review we have focused on the link between insulin analogue exposure and breast cancer development by including only in vitro studies that have used breast cancer cell lines or animal and patient derived studies that have focused on mammary gland tumor development specifically.

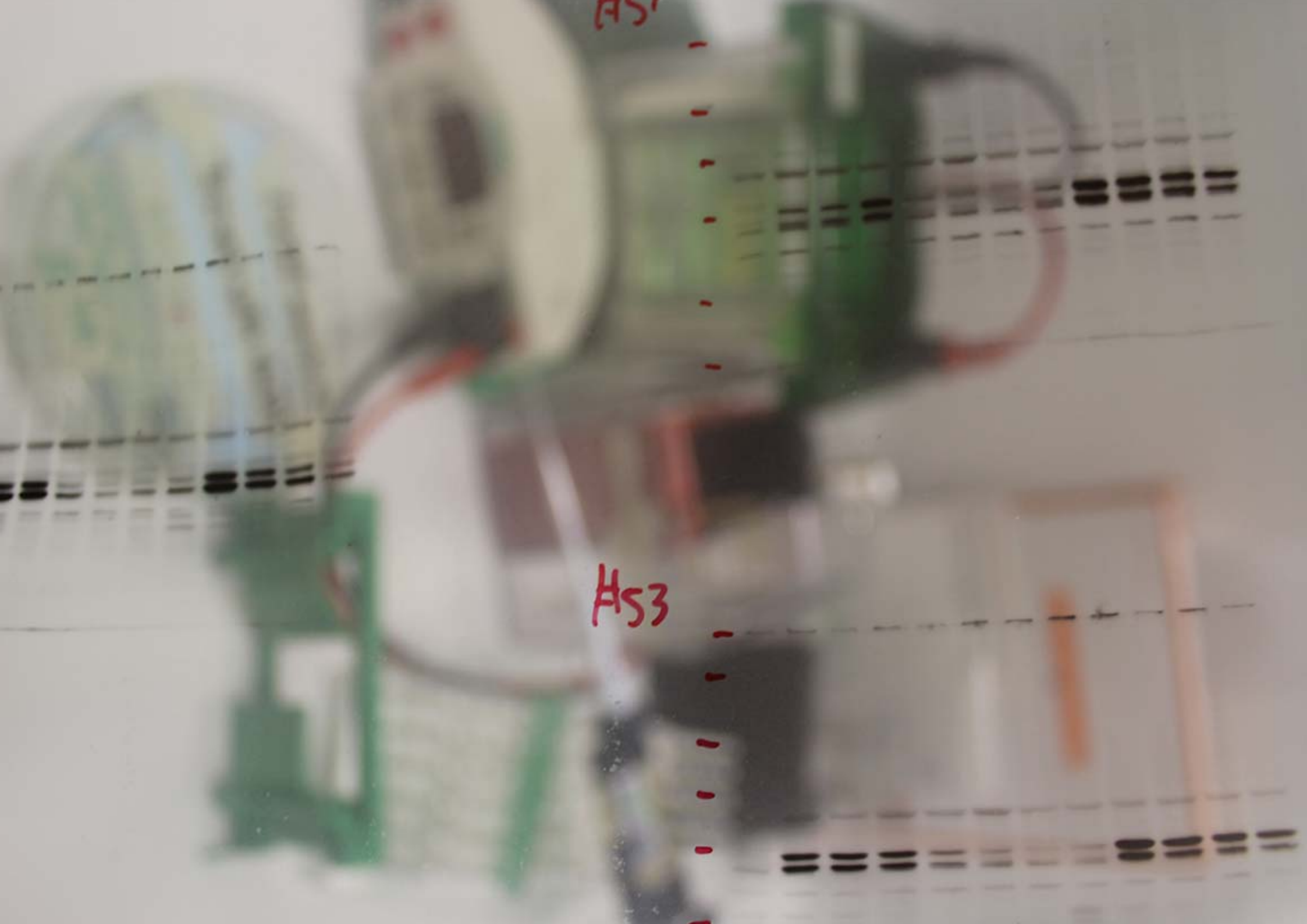
An in vitro study is described in **chapter 3**, in which a new cell model was used to determine the mitogenic activities of insulin analogues. A stable knockdown was combined with a retroviral overexpression system by which the MCF7 human breast cancer derived cell model only expressed one of the involved receptors (IRA, IRB or IGF1R). Exposure experiments have been performed including all commercial insulin analogues as well as regular human insulin, AspB10 and IGF1 as reference compounds. Several exposure times as well as concentrations have been included. Both a mechanistic information was gathered using the protein quantification methods as a read out as well as functional assays determining the direct proliferative effects of these compounds.

In **chapter 4**, the same cell model was used as in chapter 3. This time a full transcriptomic analysis was performed using micro-arrays. The genes involved in insulin analogue mitogenic signaling were identified. The predictive potential of these mitogenic gene classifiers were tested using all commercially available analogues. Furthermore validation and possible clinical relevance was assessed by testing the gene expression levels of these classifiers in different models, including primary human mammary cells and mouse mammary glands.

An in vivo experiment was carried out using the $p53^{R270H/+}$WAPCre mouse model. This model has a human relevant mutation in the tumor suppressor p53 gene by which it will develop spontaneous mammary gland tumors. In **chapter 5**, we determined if chronic insulin analogue treatment would affect the tumor latency time. Furthermore a phospo-proteomic analysis on the tumors was performed to detect treatment specific differences between the insulin analogue induced tumors.

These tumors were further characterized in **chapter 6** using next generation sequencing. We anticipated that the full transcriptomic analysis would shed light on how these tumors have developed and to pick up treatment specific tumor-related effects. Furthermore a mutational analysis was performed on these tumors.

Finally, **chapter 7** provides a general discussion on the results obtained in our studies and on the implications for future research.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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:

B. ter Braak*, H. K. Bronsveld*, Ø. Karlstad, P. Vestergaard, J. Starup-Linde, M. T. Bazelier, M.L. De Bruin, A. de Boer, C.L.E. Siezen, B. van de Water, J.W. van der Laan, M.K. Schmidt

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

Submitted, 14-02-2015, Diabetes care

◀ 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.

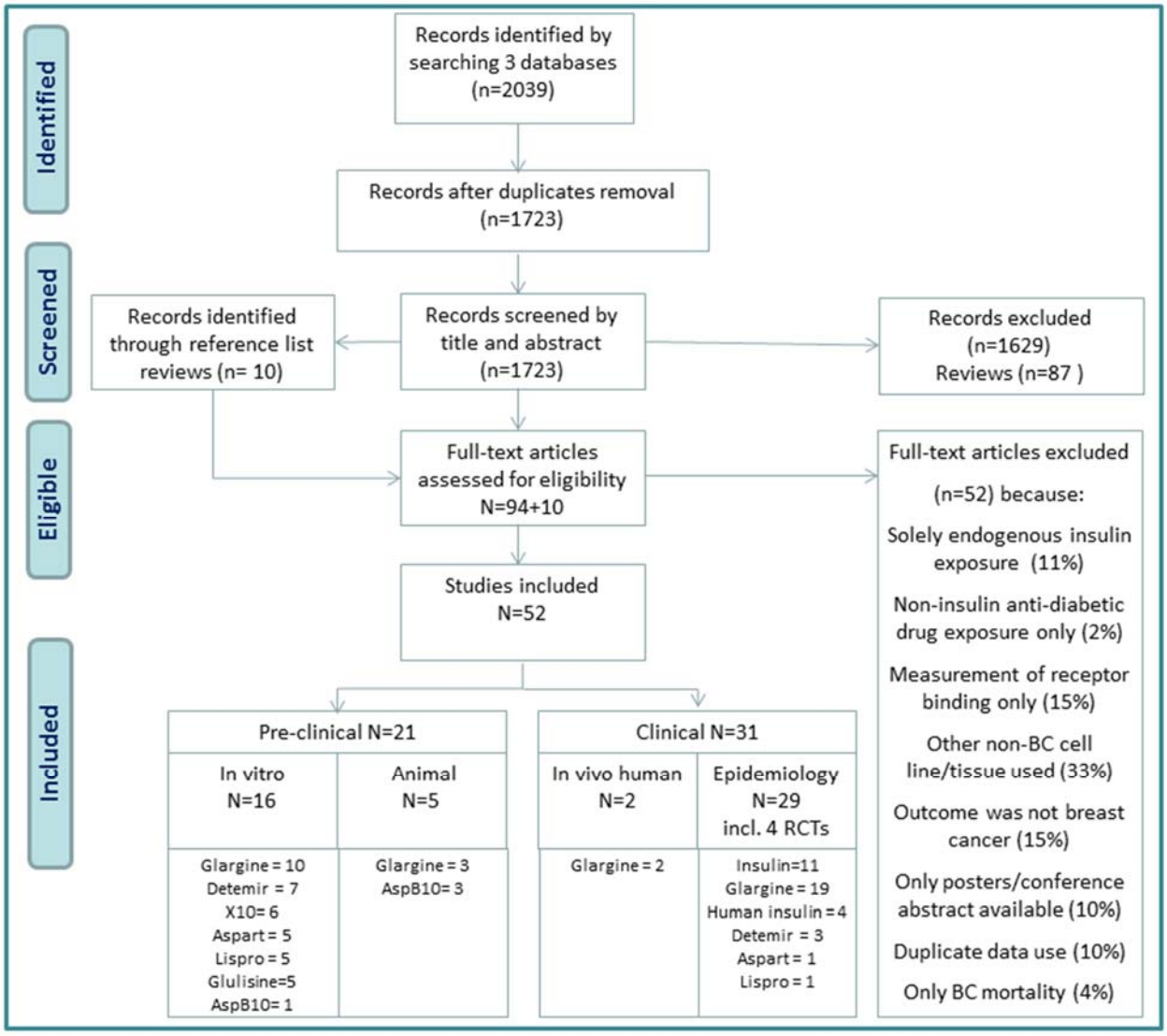

Fig. 1 Flow chart of study identification and study selection process.

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.

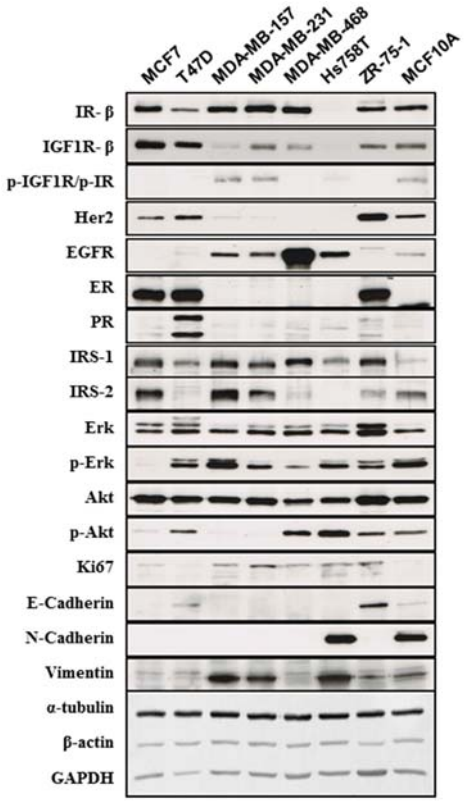

Fig. 2 Protein expression profiling of eight commonly used human breast cell lines. Receptor levels and signalling molecules downstream of the INSR/IGF1R signalling pathway have been quantified. Furthermore some breast cancer subtype markers have been used to further characterize these cell lines that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

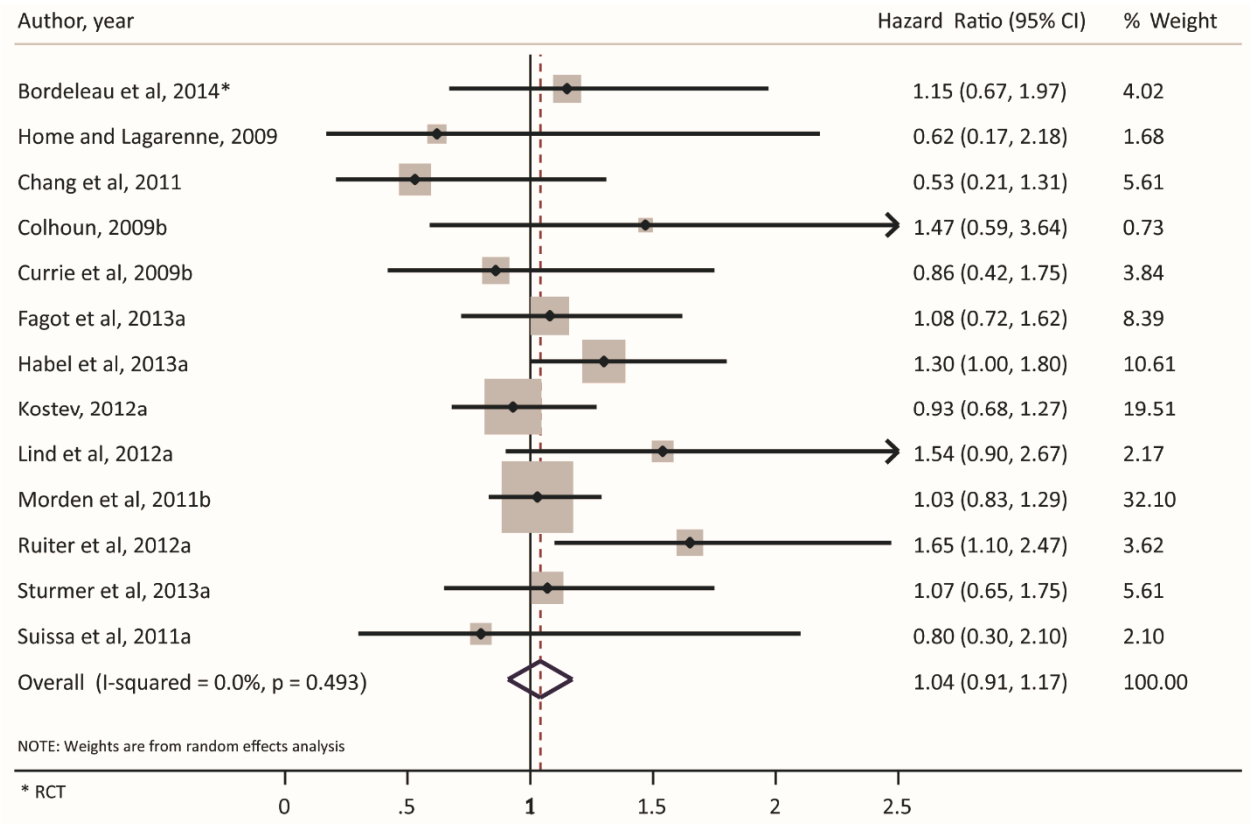

Fig. 3 Forest plot reported hazard ratios for risk of breast cancer among insulin glargine users.

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].

Acknowledgement

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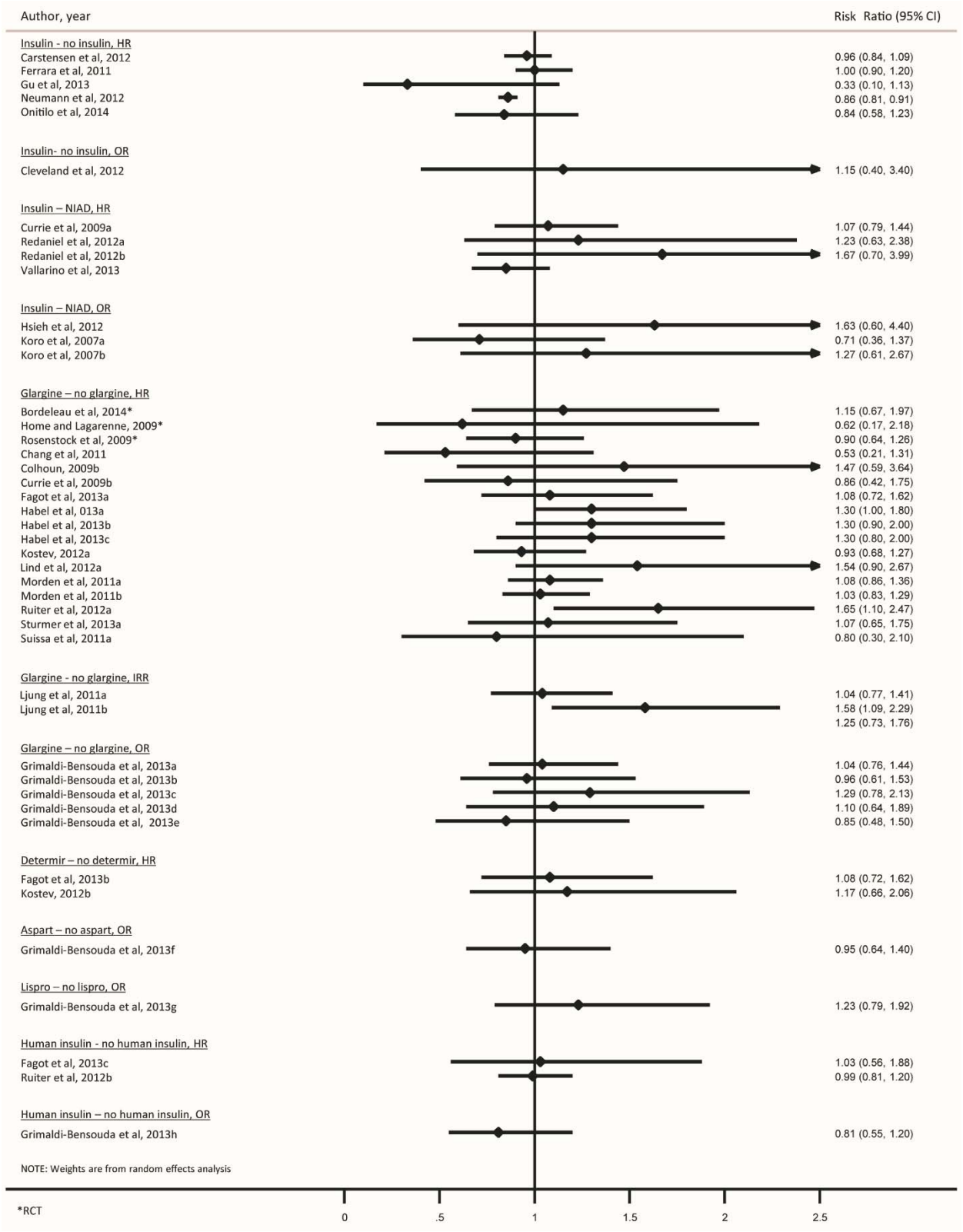
Duality of interest

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

Chapter 3

Classifying the adverse mitogenic mode of action of insulin analogues using a novel mechanism-based genetically engineered human breast cancer cell panel

Highlights

- IGF1R is transmitting most of the insulin analogue induced proliferative signalling
- Glargine strongly induces mitogenic signalling cascades in a similar extent as AspB10
- All other commercial insulin analogues as well as the two metabolites of glargine have a mitogenic potential similar to insulin

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Classifying the adverse mitogenic mode of action of insulin analogues using a novel mechanism-based genetically engineered human breast cancer cell panel

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◀ IN THE PICTURE

SRB proliferation assay plates. The SRB technique is used to quantify proteins in a sample, which is used as a measure for the total amount of cells. A high intensity of pink colour indicates a high number of cells present in the sample. By doing dose response experiments or measure sequential days, the growth potential of cells under specific conditions can be determined. A SRB-based screen will generate many plates. An estimated three hundred 96-well plates were used in this research.

◀ IN BEELD

SRB proliferatie analyse platen. De SRB techniek wordt gebruikt om het totaal aan eiwitten te kwantificeren, hetgeen een maat is voor het totaal aantal cellen in een monster. Hoe rozer het welletje hoe meer cellen aanwezig zijn. Door op verschillende dagen te meten kan de groeipotentiaal van cellen bepaald worden onder specifieke condities (bijv. aanwezigheid van een bepaalde stof). Een analyse gebaseerd op deze methode resulteert al snel in veel platen. Ongeveer driehonderd 96-welletjes platen zijn gebruikt in dit onderzoek.

Abstract

Insulin analogues are widely used in clinical practice. Modifications on the insulin molecular structure can affect the affinity and activation towards two closely related receptor tyrosine kinases: the insulin receptor (INSR) and the insulin-like growth factor 1 receptor (IGF1R). A switch towards higher IGF1R affinity is likely to emphasize mitogenesis rather than glucose metabolism. Relevant well-validated experimental tools to address the insulin analogue activation of either INSR or IGF1R are missing. We have established a panel of human MCF-7 breast cancer cell lines either ectopically expressing the INSR (A or B isoform) in conjunction with a stable knockdown of the IGF1R or ectopically expressing the IGF1R in conjunction with a stable knockdown of the INSR. In these cell lines, we systematically evaluated the INSR and IGF1R receptor activation and downstream mitogenic signalling of all major clinical relevant insulin analogues in comparison with insulin and IGF1R. While most insulin analogues primarily activated the INSR, the mitogenic activation pattern of glargine was highly similar to IGF1 and insulin AspB10, known to bind IGF1R and induce carcinogenesis. Yet, in a long-term proliferation assay, the proliferative effect of glargine was not much different from regular insulin or other insulin analogues. This was caused by the rapid enzymatic conversion into its two metabolic active metabolites M1 and M2, with reduced mitogenic signalling through the IGF1R. In summary, based on our new cell models, we identified a similar mitogenic potency of insulin glargine and AspB10. However, rapid enzymatic conversion of glargine precludes a sustained activation of the IGF1R signalling pathway.

Keywords: insulin glargine, mitogenic, human breast cancer, IGF1R

Introduction

Both type 1 and type 2 diabetic patients benefit from insulin injections. Modifications to the structure of insulin have changed its absorption, distribution, metabolism and excretion (ADME) characteristics. Fast acting analogues (e.g. aspart, lispro and glulisine) have been developed that are more readily absorbed from the injection site compared to regular insulin, intended to supply bolus level of insulin needed after a meal. Long acting analogues (e.g. glargine and detemir) are released slowly and steady from the injection site, intended to supply basal level of insulin activity during the nocturnal period.

Two decades ago, the fast acting insulin AspB10 [107] was found to induce tumor formation in rats [108] and therefore never entered the market. Yet it raised speculations on the cancer risk of insulin analogues. Therefore, all new insulin analogues require testing for their mitogenic and carcinogenic potential in *in vitro* and *in vivo* assays, which have severe limitations. While insulin glargine (Lantus) was negative in the 2-year rodent carcinogenicity studies, epidemiological studies on cancer incidence based on diabetic patient data are conflicting. While one camp

suggests no relation between the use of insulin glargine and the occurrence of any cancer [18, 109] [19] [67] [86], others found an association with (breast) cancer development [16] [70] [17] [76]. The relevancy of life-long bioassays in animals for these type of products can be questioned.

Similarly, *in vitro* studies have led to contradictory findings regarding the mitogenic potency of insulin analogues. Comparisons are difficult to make as sometimes no distinction was made between activation of the different isoforms of the insulin receptor, different cell lines, culture conditions and/or treatment regimens were used. Some studies indicate a clear increased mitogenic potency of insulin glargine in comparison with regular human insulin [37, 43, 45, 110-117] whereas others found no difference in mitogenic potential [118-126]. *In vitro* studies have shown that insulin glargine has, like AspB10, an increased binding affinity for the IGF1R [114] [127]. A prolonged occupancy time for the insulin receptor A isoform (IRA, the insulin receptor isoform without exon 11) might also contribute to the increased mitogenic effect of some insulin analogues [113]. A major drawback of these studies is the presence of both INSR and IGF1R in the cells, precluding the relative contribution of these receptors to the downstream mitogenic signalling and ultimate increased cell proliferation.

Here we applied a unique approach by integrating receptor overexpression studies with RNA interference techniques to selectively assess the contribution of the insulin and IGF1 receptors in insulin analogue-based signalling. Thus we established a complete novel panel of human MCF-7 breast cancer cell lines that either ectopically expresses the IGF1R combined with stabile lentiviral shRNA-based knockdown of IR, and reversely cell lines ectopically expressing IRA or IRB with stabile shRNA-based knockdown of IGF1R. The different human cell lines were systematically treated with physiological relevant concentrations of insulin and various clinical relevant insulin analogues including insulin glargine, the metabolites of glargine (M1 and M2), AspB10, aspart, glulisine, lispro, detemir as well as IGF1. The pro-mitogenic signalling cascade activation and proliferation potential were systematically determined for the different insulin analogues. The effect in these breast cancer cell lines was compared with the *in vivo* activation of the pro-mitogenic signalling in the mouse mammary gland.

Materials and Methods

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) (Cell Signalling Technology, Danvers, MA, USA), mouse anti-IGF1Rβ and rabbit anti-IRβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-tubulin (Sigma-aldrich, St. Louis, MO, USA) were commercially purchased. Conjugated secondary antibodies included anti-mouse

horseradish peroxidase (HRP) and anti-rabbit HRP (Jackson ImmunoResearch, West Grove, PA, USA).

Human insulin (Humalin Lilly, Fegersheim, France), the insulin analogues (table 1) and IGF1 (Increlex, Ipsen Pharma, Boulogne-Billancourt, France) were commercially purchased. The two main metabolites of insulin glargine (M1 and M2) were kindly provided by Dr. N. Tennagels (Sanofi-Aventis, Frankfurt, Germany). Insulin AspB10 was kindly provided by Dr. B. Falck Hansen (Novo Nordisk, Copenhagen, Denmark). To maintain the stability of these analogues 1000x concentrated stock solutions were prepared in their original vehicle solutions.

Table 1. Insulin analogues used in this study with additional information.

Generic name	Brand name/ CAS #	Mutations made to the insulin molecular structure	Company abbreviation	Company
aspart	NovoRapid®/116094-23-6	B28Asp human insulin	B28Asp	Novo- Nordisk, Bagsvaerd, Denmark
AspB10	Not marketed	B10Asp human insulin	X10	Novo- Nordisk, Bagsvaerd, Denmark
detemir	Levemir®/ 169148-63-4	B29Lys (epsilon-tetradecanoyl), desB30 human insulin	NN 304	Novo- Nordisk, Bagsvaerd, Denmark
glargine	Lantus®/ 160337-95-1	A21Gly,B31Arg,B32Arg human insulin	HOE 901	Sanofi-Aventis, Paris, France
glulisine	Apidra®/ 207748-29-6	B3Lys,B29Glu human insulin	HMR1964	Sanofi-Aventis, Paris, France
lispro	Humalog®/ 133107-64-9	B28Lys, B29Pro human insulin	LY275585	Eli Lilly, Indianapolis, IN, USA
metabolite 1 of glargine	Not marketed	A21Gly human insulin	M1	Sanofi-Aventis, Paris, France
metabolite 2 of glargine	Not marketed	A21Gly, des-B30Thr human insulin	M2	Sanofi-Aventis, Paris, France

Cell culturing

MCF-7 cells (ATTC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin (Invitrogen), which is referred to as complete medium. Cells were grown in cell culture flasks (Corning) till 70-80% confluence was reached. For all the assays described in this manuscript, cells with a passage number between 2 and 6 after transduction were used.

Phoenix retroviral overexpression

pBABE-zeo (containing zeomycin resistance gene) and pBABE-IRB (containing both a zeomycin resistance gene and the cDNA of the human *Irb* gene) were kindly provided by Prof. G. R. Guy

(Signal Transduction Laboratory, Institute of Molecular and Cell Biology, Singapore). The pNTK2-neo plasmid containing the neomycin resistance gene and pNTK2-HIR (a vector with a neomycin resistance gene and the cDNA of the human *Ira* gene) were kindly provided by Prof. A. Ullrich (Max Planck institute of Biochemistry, Germany). IRA and IRB encoding retroviruses were produced by transfection with the previously mentioned plasmids into the phoenix amphotropic packaging cells described by Swift et al. [128]. MCF-7 cells were infected with freshly harvested retroviral supernatant, cultured for two days on complete medium and selected with 400 µg/ml neomycin (for MCF-7 IRA) or 400 µg/ml zeomycin (for MCF-7 IRB) for one week. Multiple clones were generated and with western blotting the highest stable IR overexpressing MCF-7 lines were selected. Stable IGF1R over expressing MCF-7 cells (using the pMSCV-neo-IGF1R vector) were described previously by us [129].

Lentivirus shRNA knockdown

For the preparation of stable shIR and shIGF1R cell lines, MCF-7 wt, MCF-7 IGF1R, MCF-7 IRA or MCF-7 IRB cells were transduced by using lenti-viral shRNA vectors (Sigma-Aldrich), kindly provided by Prof. R. Hoeben, Leiden University Medical Center (LUMC). Several shRNA constructs per receptor were tested. TRCN0000000424, target sequence: GCTGATGTGTACGTTCCTGAT (shIGF1R) and TRCN0000000380 target sequence: GTGCTGTATGAAGTGAGTTAT (shIR) showed the highest (>90%) knockdown efficiency (data not shown), these constructs were used to knockdown the receptors. Cells were selected using puromycin (4 µg/ml) for one week.

Cell treatment

Cells were seeded in 6-well plates at a confluence of 60-70% in complete medium. The next day, cells were starved overnight with 1% FBS containing RPMI medium, followed by 2 hours of serum deprivation with serum free medium (SFM). Cells were exposed to insulin and its analogues at a given dose in SFM for 30 minutes, directly followed by cell lysis.

Western blot analysis

For the general materials and methods of the cell lysis and Western blot analysis we refer to Zhang et al., 2011 [129]. Total protein was separated on a 7.5% (IR, IGF1R and p-IGF1R) or 10% (Erk1/2, p-Erk, Akt, p-Akt). Blocking was performed at room temperature for 1 h in either 5% (w/v) bovine serum albumin BSA (for Erk, Akt, p-Erk, p-Akt and tubulin) or 5% (w/v) not-fat dried milk (for IGF1Rβ, IRβ and p-IGF1R/p-IR) dissolved in washing buffer (100 mM Tris, pH 7.4, 500 mM NaCl, 0.05% Tween 20). PVDF membranes were exposed to Pierce® ECL Western blotting substrate (Thermo Scientific, Rockford, IL, USA) and proteins were visualized by placing the membrane in contact with standard X-ray film (GE Healthcare, Little Chalfont, England). Thereafter the film was developed with a Kodak X-omat 1000 processor. A Cy-5 conjugated secondary antibody, diluted 2000 times in 1% BSA/washing buffer, was used for the tubulin

stained membrane, which was scanned using a Typhoon 9400 imager. For the quantification of the western blot results, all bands were quantified using ImageJ software (ImageJ, 1.43u). The intensity of each band was divided by the tubulin intensity of that same sample and subsequently divided by the corrected intensity of the endogenous control of the same blot. Hierarchical clustering on these values was performed using Spotfire (Spotfire®DecisionSite®9.1.2, Version 20.5.1039) with a Euclidean distance.

Cell proliferation

Cells were seeded with 10,000 cell/well in 96-well plates in complete medium and incubated overnight. Prior to the insulin (analogue) or IGF1 stimulation, cells were starved for two days in phenol red free RPMI 1640 medium (Gibco) supplemented with 5% charcoal/dextran-stripped FBS (CDFBS) (HyClone Laboratories, Thermo Scientific, Logan, USA), devoid of steroid hormones and very low (0.18 pM) insulin levels. Starved cells were stimulated with insulin, its analogues or IGF1 with a given concentration in triplicate and allowed to proliferate for four days.

The sulforhodamine B (SRB) colorimetric assay [130] was used to determine the relative number of cells. This assay was described previous by our group [129].

IR PCR and AVRII restriction analysis

Cells were harvested using a cell lifter, followed by RNA isolation using the RNeasy ® Plus mini kit (Qiagen GmbH, Germany). From the isolated RNA, cDNA was synthesized using a RevertAid H minus first strand cDNA synthesis kit (Fermentas international Inc.). Primers were designed around exon 11 of the IR (5'-TGGATTATTGCCTCAAAGGGC-3' and 5'-GAGACGCAGAGATGCAGC-3'). The Phire polymerase chain reaction (PCR) kit (Finnzymes) was used to generate a 691 bp (IRB) or a 655 bp (IRA) amplicon. Thereafter, the samples were cut using the AVRII restriction enzyme (Bioke, New England Labs). This restriction enzyme makes an asymmetrical cut within the exon 11 of IRB, cleaving the 691 bp amplicon in a 435 bp and 256 bp fragment. The PCR/restriction enzyme mix was loaded on a 2% agarose gel and imaged with a UV transilluminator.

Insulin glargine metabolism in vitro

I*n vivo*, insulin glargine is rapidly processed into two metabolically active metabolites (A21-Gly-insulin as M1 and A21-Gly-des-30G-Thr-insulin as M2) from the injection site [131]. The extent of glargine degradation in our SRB assay set-up was determined. MCF-7 cells were starved for two days with 5% CDFBS containing RPMI medium and stimulated for 0, 6, 24, 48, 72 or 96 hours with 100 nM insulin glargine (diluted in 5% CDFBS). 50 µL of supernatant was taken at the given time points and directly added to 50 µL of HCl (100 mM). Samples were kept frozen (-20 ˚C) until the analysis. Using immunoaffinity columns glargine, M1 and M2 were extracted and quantified by a specific liquid chromatography mass-tandem spectrometry assay [132]

Insulin glargine metabolism in vivo

Ten female $p53^{+/R270H}$ mice (Jackson laboratories (Bar Harbor, ME)) got a single subcutaneous injection with 100 nmol/kg of glargine, 1 hour (n=5) and 2 h (n=5) after injection mice were sacrificed, at both time points also LANTUS-vehicle injected mice were sacrificed. Two mammary glands per mouse were snapfrozen in liquid nitrogen and used in a Western blot analysis to measure activation of mitogenic pathways upon glargine treatment. Blood was extracted in K2-EDTA coated blood collection vials (Sarstedt) with 20 µl/ml protease inhibitor cocktail and put immediately on ice. Within 1.5 hours the tubes were centrifuged (1500g, 10˚C for 10 min) and transferred to regular eppendorf tubes. Samples were stored at -20C and within a month measured. Using the before mentioned liquid chromatography mass-tandem spectrometry assay the M1, M2 and glargine concentrations have been determined in the plasma.

Statistical analysis

Each SRB data point represents the average of corrected proliferation data from five independent experiments. Within such an experiment each average SRB absorbance value was derived from triplicate samples. For each experiment the average SRB was corrected against the unstimulated (0% SRB proliferation) and the maximal, 333 nM stimulated sample (100% SRB proliferation). A non linear sigmoidal dose response (with variable slope) curve was used to fit the data and retrieve the EC50 values.

Results

MCF-7 wt expresses biologically active IR and IGF1R and downstream pathways of these receptors are intact

First we selected the most appropriate cell line for our model. As IGF1 and insulin AspB10 signalling has been associated with breast cancer [133] [108], a human breast cancer cell line was a logical choice. To ensure the presence of both the IR and IGF1R signalling pathways we first evaluated the IR and IGF1R protein levels in 41 human breast cancer cell lines (data not shown). Five were selected that expressed both the IR and IGF1R at relatively high levels (HCC38, MCF-7, MDA-MB-231, SUM44PE and UACC812). SUM44PE and UACC812 are known to express high levels of ErbB2, a dominant oncogene for mitogenic signalling, and were therefore discarded. The MCF-7 cell line showed the highest overall IR and IGF1R levels as compared to other breast cancer cell lines and since this is a well defined cell model, it was selected to further study the responsiveness to insulin and IGF1 (fig 1). IGF1 caused strong phosphorylation of the IGF1R and subsequent activation of the canonical pathways downstream of these receptors (MAPK/Erk and PI3K/Akt); also insulin caused activation of these mitogenic programs, with mild phosphorylation of the IR at high concentrations.

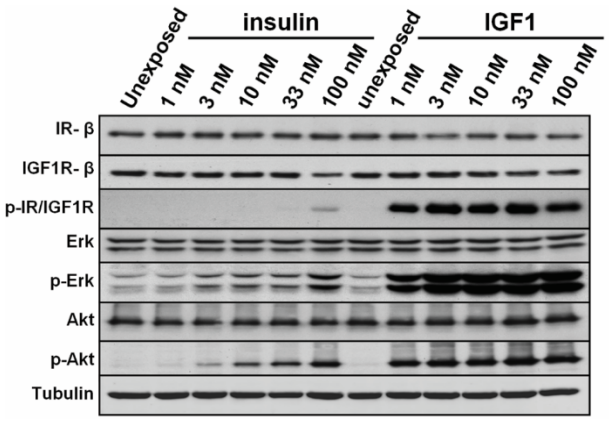

Fig 1 Dose dependent activation of receptors and downstream signalling pathways in MCF7 cells. Treatment of MCF-7 cells with either insulin or IGF1 caused a concentration dependent activation of the IR and IGF1R, respectively. MCF-7 was stimulated for 30 minutes with human Insulin and IGF1 (at 1, 3, 10, 33 and 100nM). IR, IGF1R, p-IR/IGF1R, Akt, p-Akt, Erk, p-Erk and tubulin protein levels are determined with Western blots.

IRA, IRB, IGF1R over-expressing MCF-7 cell lines with knockdowns of IR or IGF1R have varying IRA/IRB/IGF1R levels and the transfections did not affect the Erk or Akt levels.

We wanted to selectively study the effect of insulin analogues on IR (IRA and IRB) and IGF1R. Therefore, we generated MCF-7 cell lines with increased IR or IGF1R receptor levels. Importantly, we subsequently created stable lentiviral-based knockdown of the IR, for the IGF1R over expressing cell line (MCF-7 IGF1R) and knockdown of the IGF1R in the IRA and IRB over expressing cells (resp. MCF-7 IRA and MCF-7 IRB). This resulted in a unique panel of cell lines to selectively study IRA, IRB and IGF1R signalling (fig 2a).

The lentiviral shCtrl and the pBABE-empty did neither affect receptor levels nor the total levels of Akt or Erk. Overexpression IRA/IRB/IGF1R (all around 200% in comparison with MCF7 wt) and knockdown of IR did not have a clear effect on the activation of Akt and Erk (fig 2b-f). But shIGF1R led to a strong decrease in Erk activation, without affecting Akt. The IRA and IRB overexpressing cell lines did show activation of the receptor by insulin and subsequent subtle Erk and Akt phosporylation; this was not observed in the wildtype MCF-7 cell line. The IGF1R overexpressing MCF-7 cell line strongly responded to IGF1 stimulation in terms of IGF1R, Erk and Akt phosphorylation. Some residual IGF1R expression remained in the MCF-7 IRA shIGF1R and MCF-7 IRB shIGF1R cell lines. The antibody that was used to detect the beta-subunit of the IR can not discriminate between the two isoforms IRA or IRB of the insulin receptor. To qualify the insulin receptor isoforms in the established cells a PCR was performed on cDNA with primers that amplify around exon 11 of the IR, resulting in an amplicon of 691 bp (in case of IRB cDNA) or 655 bp (in case of IRA cDNA). To confirm the presence of exon 11 in the IRB amplicon an AVRII restriction was performed, cleaving the IRB amplicon in two asymmetric pieces of 435 bp and 256 bp. The MCF-7 wt cells expressed both isoforms of the insulin receptor. Further, it was confirmed that the appropriate isoform is expressed in the different cell lines, with some residual expression of IRA in the MCF-7 IRB shIGF1R cell line (data not shown).

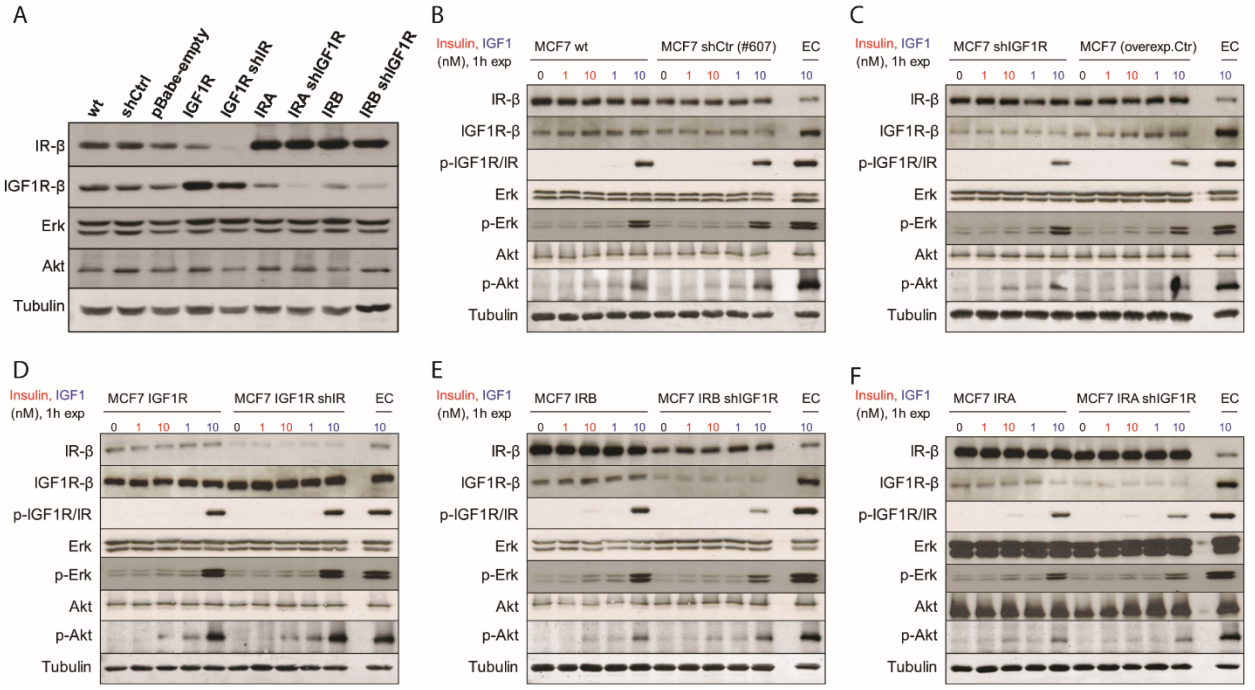

Fig 2 Generation of a panel of MCF7 cell lines with different receptor levels with an active downstream signalling pathway. A) Western blot analysis on protein levels (IR, IGF1R, Erk and Akt) of the established cell lines (MCF-7 wt, MCF-7 shCtrl, MCF-7 pBABE-empty, MCF-7 IGF1R, MCF-7 IGF1R shIR, MCF-7 IRA, MCF-7 IRA shIGF1R, MCF-7 IRB, MCF-7 IRB shIGF1R). B-F), 1 hr exposure of 10 different cell lines to insulin and IGF1 to measure activation of downstream signalling cascades. This graph indicates that the ectopic expression and knockdown of the IR and IGF1R cell lines have a clear effect on the IR and IGF1R levels and that this has a clear effect on the activation of mitogenic pathways. The cell lines transfected with the control (empty) vectors (shCtrl or overexp. Ctrl pBABE-empty) show protein levels comparable with MCF-7 wt. EC is endogenous control (MCF7 IGF1R stimulated with 10 nM of IGF1) loaded on every blot to be able to make a fair comparison between different blots.

Insulin glargine activates Akt and Erk similar to AspB10 and IGF1

Next, we systematically investigated the mitogenic signalling activity of the various insulin analogues in three of the established cell lines: MCF-7 IRA shIGF1R, MCF-7 IRB shIGF1R and MCF-7 IGF1R shIR (from now on referred to as MCF-7 IRA, MCF-7 IRB and MCF-7 IGF1R respectively). Most insulin analogues showed a concentration dependent activation of the receptors and the downstream Akt and Erk pathways, except IGF1 and detemir (fig 3). IGF1 demonstrated maximal activation of the IGF1R already at the lowest (10 nM) concentration. Despite the fact that IGF1 has a very low binding affinity towards the IRB [114] [127], in the MCF-7 IRB cell line and after IGF1 stimulation some activation of Erk and Akt was observed. This might be due to an incomplete knockdown of IGF1R in these cells. Insulin stimulation led to activation of the IRA and IRB, while IGF1R was only activated at the highest concentration (100 nM) of insulin. The Erk activation follows the activation of these receptors quite strictly, yet the phosphorylation of Akt was typically stronger via the IGF1R. Like IGF1, AspB10 was able to activate the IGF1R and the downstream signalling cascade at low concentrations. Low concentrations of glargine also led to a strong activation of the IGF1R, Erk and Akt, mimicking the AspB10 response. In contrast, aspart

and glulisine have a similar receptor and downstream activation pattern that is most similar to that of regular insulin. Lispro seems to be able to activate the IGF1R slightly more, but the phosphorylation of Akt and Erk is not more induced compared to regular insulin stimulation. Detemir only mildly activated the receptors, and only a very slight Erk activation was observed via the IRB. Overall, the IGF1R seems to have the strongest effect on mitogenic (Erk/Akt) signalling, whereas the IRB is hardly involved. These findings are in agreement with literature [134] [135] [136]. To allow for a more unbiased comparison of the effects of insulin homologues we quantified all the different signalling responses followed by hierarchical clustering of the data (fig 4). The activation pattern of insulin glargine stimulation via the IGF1R and IRA clustered with the activation pattern of AspB10 and IGF1. In contrast, aspart, lispro and glulisine showed an activation pattern similar to that of regular insulin. Insulin detemir was the least potent compared to regular insulin in activating mitogenic pathways at the tested concentrations; which fits with its *in vivo* potency (1 unit of human NPH insulin is 6 nmol vs 1 detemir unit is 24 nmol). [137] In conclusion, insulin glargine appears to activate the Erk (MAPK) and Akt (PI3K) through IGF1R signalling in a similar extent as AspB10.

The proliferative effect of insulin glargine is similar to that of human insulin

Next we evaluated whether the similarity of the activation of the mitogenic signalling by insulin glargine, AspB10 and IGF1 correlated with cell proliferative effects of these insulin analogues. For this we determined the proliferative capacity in our three cell lines (MCF-7 IRA, MCF-7 IRB and MCF-7 IGF1R) using a broad concentration range (0.01 to 333 nM). All insulin analogues showed a clear concentration dependent proliferative effect that followed a sigmoidal dose response (fig 5a-c). Also regular insulin had a clear intrinsic proliferative effect. MCF-7 IGF1R cells showed the highest response to insulin analogues; again, the MCF-7 IRB showed the lowest response. In all cell lines AspB10 and IGF1 had the lowest EC50 values (fig 5d). Aspart, lispro and glulisine had very similar proliferation effects to regular insulin. Insulin detemir showed the lowest effect on proliferation in all cell lines. Importantly and unexpectedly, insulin glargine had comparable EC50 values to regular insulin, indicating that initial strong mitogenic signalling mediated by insulin glargine exposure did not lead to an increased mitogenic potential compared to regular insulin.

Insulin glargine is rapidly metabolised

To clarify the discrepancy between insulin glargine mitogenic signalling and proliferation we carefully studied the metabolism of glargine and the formation of M1 and M2 (the two main metabolites of insulin glargine). Glargine processing appeared to be very rapid: in our 5% CDFBS culture medium in the absence of cells it took less than 24 ours to process 70% of the glargine into M1 (40%) and M2 (60%), after 4 days less than 3% of the original glargine was detected (fig 6a). The addition of MCF-7 cells did not affect glargine metabolism into M1 or M2, but did influence glargine, M1 and M2 degradation. Thus, in 24 hours 50% of the insulin analogues

(glargine, M1 and M2) were degraded by the cells. The degradation speed appeared to be similar for glargine, M1 and M2.

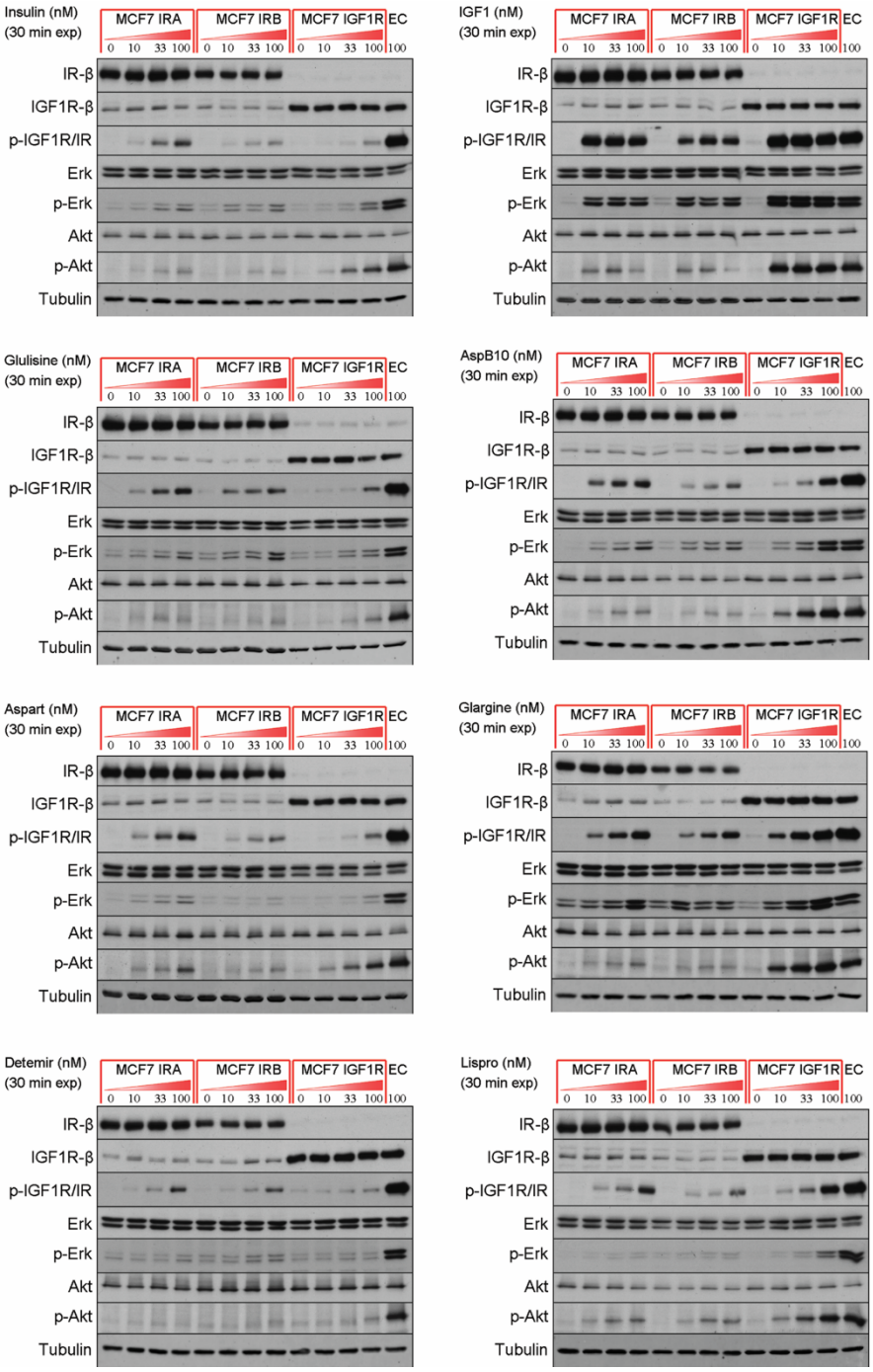

Fig 3. Stimulation of a panel of MCF7 cell lines with insulin analogues to measure downstream mitogenic signalling. Westernblot analysis of 3 different cell lines (MCF-7 IRA shIGF1R, MCF-7 IRB shIGF1R and MCF-7 IGF1R shIR) stimulated by 8 different insulin like molecules (human insulin, AspB10, IGF1, glargine, aspart, lispro, glulisine and detemir) at 4 different concentrations (0, 10, 33 and 100 nM). On each blot also an endogenous control, EC (MCF-7 IGF1R shIR stimulated with 100 nM of IGF1), was loaded. This sample was used for the quantification of the individual protein bands, to correct for any blot differences, exposure time differences etc. This experiment is performed twice and highly similar results were obtained. Only blots of the first experiments are shown.

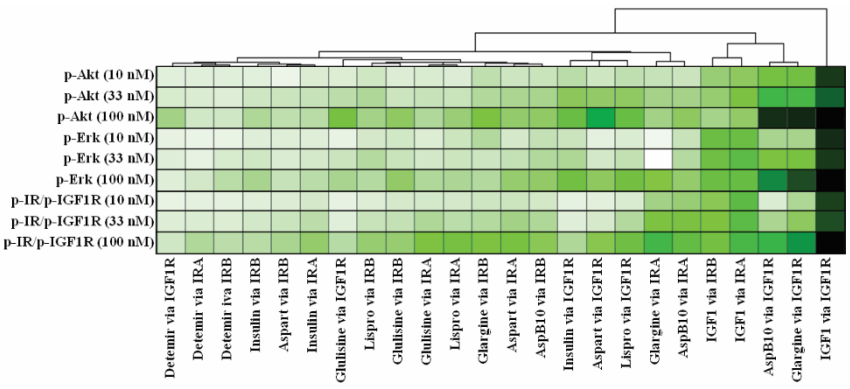

Fig 4. Hierarchical clustering (using Euclidean distance) of quantified westernblot exposure data. A White color indicates no difference in activation compared to the unstimulated sample, the increasing dark green color indicates an increasing phosphorylation of either the receptor, p-Erk or p-Akt. (n=2)

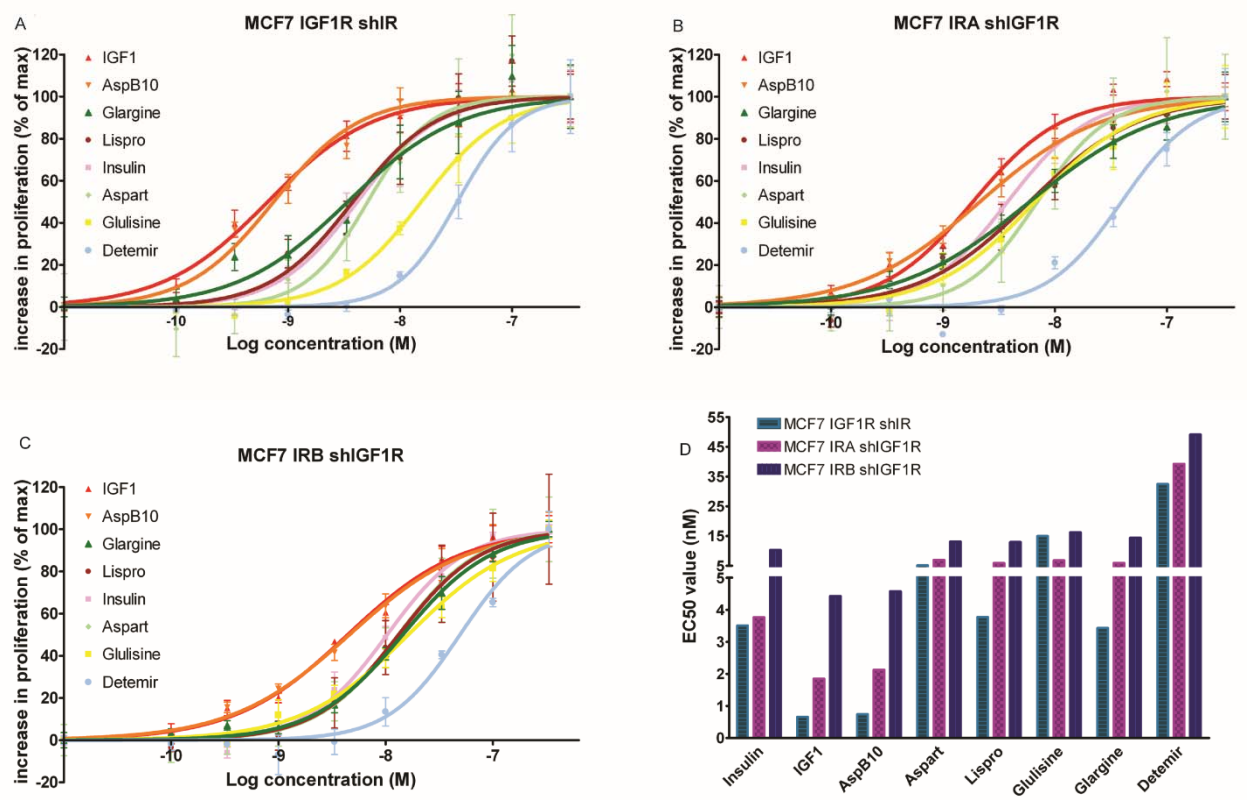

Fig 5 Dose dependent proliferative effect of all insulin analogues in panel of MCF7 cell lines a-c Effect of insulin analogues on MCF7 cell proliferation. 3 Cell lines (MCF-7 IGF1R, MCF-7 IRA and MCF-7 IRB) stimulated with 10 different insulin like molecules. Proliferation measured after 4 days of stimulation. All values are normalized against the unstimulated sample, (n=5). d) EC50 values of SRB proliferation assay.

Repeated glargine dosing lowers the EC50 reflecting IGF1

Since glargine appears to be metabolised and degraded rapidly under culture conditions we questioned whether the proliferative potential of glargine could be fully ascertained to the unprocessed form of glargine. Therefore an additional experiment was performed in which the growth factor containing medium was refreshed twice a day and compared to single growth factor stimulation (fig 6b,c). Repeated refreshing of glargine medium significantly reduced the EC50 value by more than 7-fold from approximately 23nM to about 3nM. Repeated refreshing of

IGF1 medium caused a much smaller shift from 1.8nM to 0.53nM, which is likely only due to degradation. This observation suggests that glargine has an intrinsic high mitogenic potency, while the mitogenic potential of the metabolites M1 and M2 is likely to be in the order of human insulin.

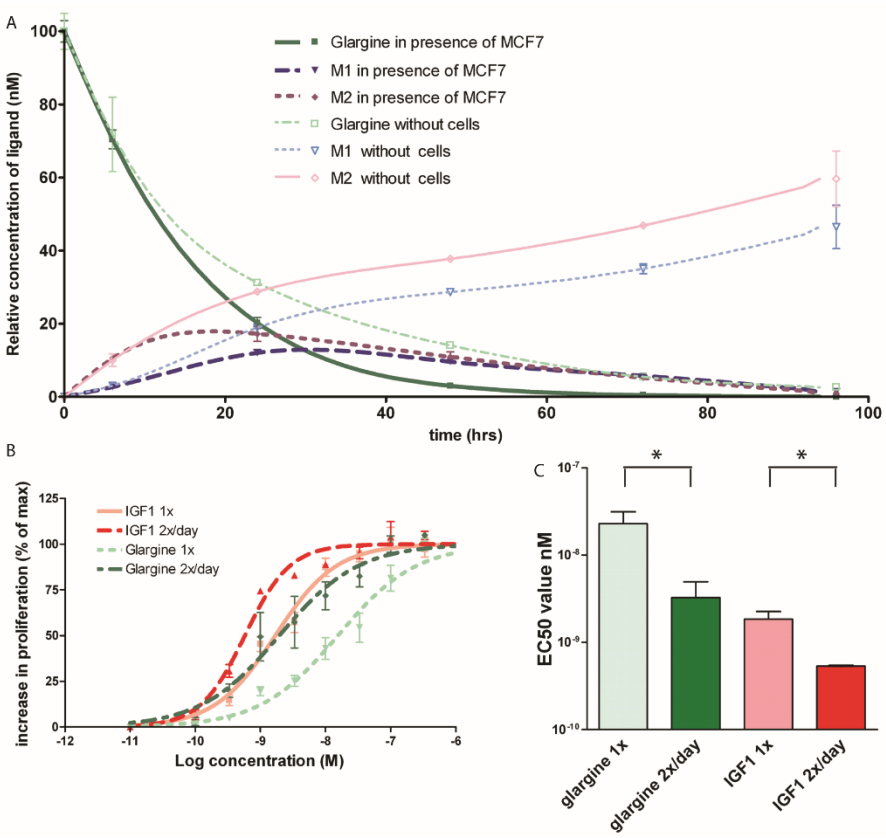

Fig 6 Glargine metabolism in long term *in vitro* assay A) Concentrations of insulin glargine, M1 and M2 over time in 5% CDFBS containing RPMI medium in the presence and absence of MCF-7 cells. B) Relative proliferation measured in MCF-7 IGF1R, stimulated once with IGF1 (light red) or glargine (light green) or the growth factor containing medium was refreshed twice a day with IGF1 (dark red) and glargine (dark green). Proliferation measured after 4 days of stimulation. All values are normalized against the unstimulated sample. N=3. C) The EC50 values of the proliferation curves. (* $p < 0.05$)

The insulin glargine metabolites M1 and M2 reflect insulin in their mitogenic potency

We next determined the mitogenic potency of the two main metabolites of glargine, M1 and M2. The activation pattern via the IRA and IRB as well as downstream Erk and Akt activation were similar for M1 and M2 and were comparable to insulin (fig 7). Importantly, little signalling was observed through the IGF1R pathway. Hierarchical clustering of the quantified Western blot data showed that the activation patterns of M1 and M2 were highly comparable to regular insulin. Finally, the proliferation assays (fig 8) confirmed that M1 and M2 have a low mitogenic potency which is comparable to regular insulin.

Glargine is rapidly metabolised into M1 in vivo but activates mitogenic signalling in the mammary gland.

Finally, we evaluated the mitogenic signalling under *in vivo* conditions. We selected glargine as the model compound since it has a major concern regarding specificity for INSR versus IGF1R. One and two hours after glargine injection, M1 and M2 levels were measured in the plasma of mice.

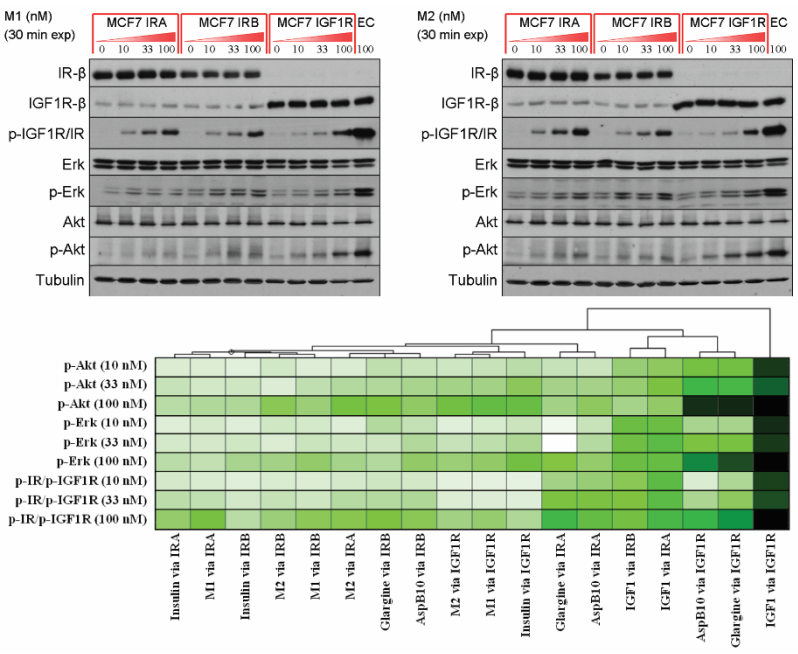

Fig 7 Metabolites of glargine show mitogenic pathway activation pattern similar to regular insulin. Westernblot analysis of 3 different cell lines (MCF-7 IRA shIGF1R, MCF-7 IRB shIGF1R and MCF-7 IGF1R shIR) stimulated by M1 and M2 at 4 different concentrations. (n=2) Only blots of the first experiments are shown. Hierarchical clustering (Euclidean distance) of quantified WB exposure data (including insulin, glargine, M1, M2, aspB10 and IGF1). The white color indicates no difference in activation compared to the non-stimulated sample, the increasing dark green color indicates an increasing phosphorylation of either the receptor, p-Erk or p-Akt. (n=2)

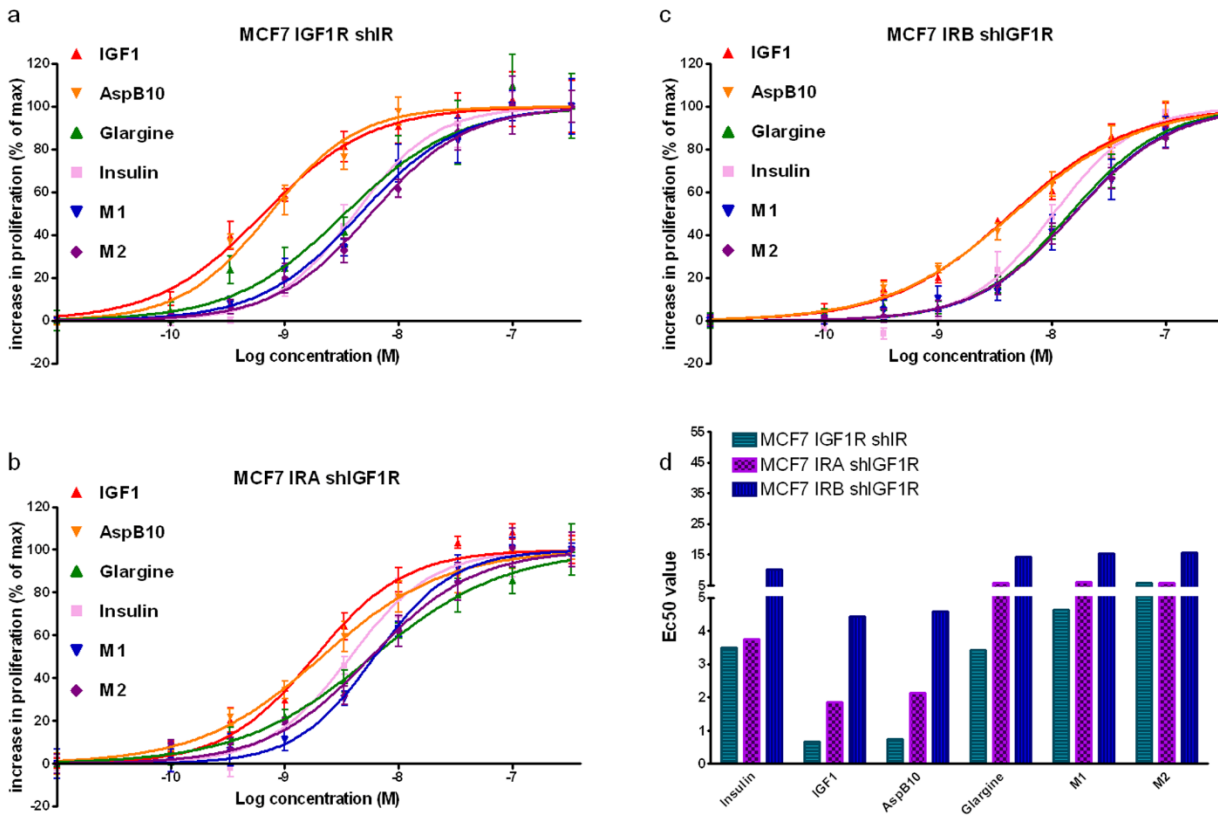

Fig 8 Metabolites of glargine show similar proliferative potential compared to regular insulin. a-c Relative proliferation measured for 3 Cell lines (MCF-7 IGF1R, MCF-7 IRA and MCF-7 IRB) stimulated with 6 different insulin like molecules. Proliferation measured after 4 days of stimulation. All values are normalized against the unstimulated sample, (n=5). d) EC50 values of SRB proliferation assay.

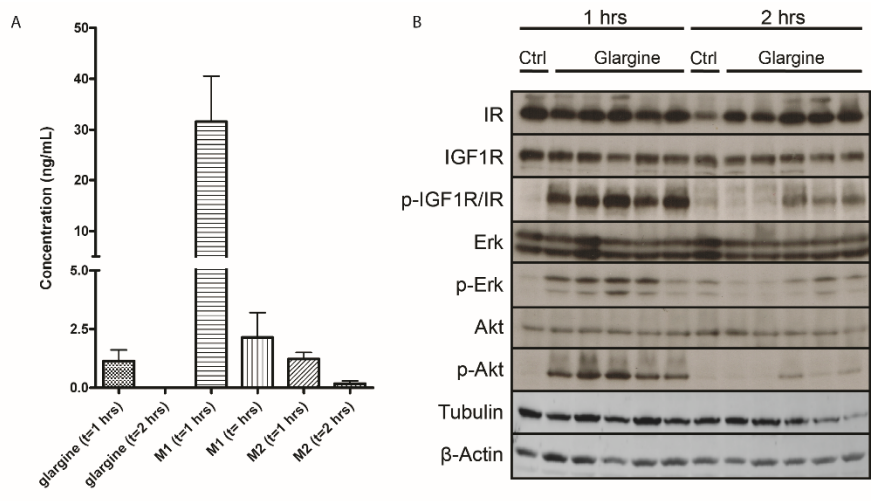

Fig. 9. Glargine metabolism and mitogenic signalling *in vivo*. Samples for glargine metabolism were taken at 1 or 2 hours after injection with glargine/vehicle. Activation of Akt and Erk in the mammary gland of mice was detected at 1 and 2 hours after glargine injections.

One hour after the injection M1 could be detected in all mice at high concentrations (Fig. 9a); only low levels of glargine and M2 could be detected. Two hours after the injections no glargine could be detected at all, very low levels of M2 (in 2/5 mice) were observed and in four out of five plasma samples M1 could be detected.This assay was used to discriminate glargine, M1 and M2 from each other and human insulin. Some cross-specificity with the mouse insulin exists for M1. This explains why small amounts of M1 were found in one vehicle control. We then evaluated the differential levels and activation of IR, IGF1R, Akt and Erk in the mammary glands of the same mice (fig 9b). While one hour after glargine injections a strong activation of both Erk and Akt was detected, after two hours hardly any activation of these mitogenic pathways was left. This indicates that glargine treatment does not provide sustained activation of the mitogenic signalling in the mouse mammary gland.

Discussion

To systematically evaluate the IR and IGF1R signalling potency of insulin analogues, in the current study a novel, simplified, well characterized cell system was established including three MCF-7 cell lines that either ectopically express the IR (A or B isoform) in conjunction with a stable shRNA-based knockdown of the IGF1R, or the ectopic expression of the IGF1R in conjunction with a stable knockdown of the IR. Through measurement of the activation of downstream signalling cascades as well as the proliferation capacity of different insulin analogues we obtained an improved definition of the mechanisms behind the cellular signalling of individual insulin analogues. Our comparative study showed that insulin glargine has a higher mitogenic potential than regular human insulin, and closely resembles that of the known mitogenic insulin AspB10, which act mainly through IGF1R. In contrast, the two main metabolites of glargine, M1 and M2, did not have such an increased mitogenic potential and have a profile closely resembling that of human insulin. Other marketed insulin analogues tested in our study, aspart, lispro, glulisine and detemir, have a mitogenic potential comparable to that of regular human insulin.

As mentioned before, there are conflicting data in the literature regarding the mitogenic potential of glargine *in vitro*. We postulate that metabolism of glargine is a very important parameter that can explain the conflicting literature data. In two studies describing long term (>2 days) proliferation assays in which the glargine containing medium was refreshed twice during the assay, a significant increased mitogenic potential of glargine was found [43] [116]. On the other hand, long term studies in which the glargine containing medium was not refreshed, and therefore significant metabolism and degradation of the compound had likely occurred, an increased mitogenic potential compared to regular human insulin was not seen [122] [124] [125, 126]. Moreover, these latter studies all used serum containing medium for their assays, making it likely that metabolizing enzymes are indeed present in the medium. Consistently, a study describing a four day proliferation assay, in which the (serum free) medium was refreshed only once, showed a non-significant increased mitogenic effect of insulin glargine compared to regular human insulin [118]. This modest effect is likely due to degradation of glargine during the two consecutive days in which the medium was not refreshed. Taken together, the conflicting results found in the literature can be explained by metabolism and degradation, as shown in our experiments, and point to a higher mitogenic potential of insulin glargine as compared to human insulin. Although we focused on the ambiguous conflicting results of glargine, some degradation is likely also the case for the other insulin analogues tested [138] [139].

Agin et al. studied how glargine is processed *in vivo* and found that on average over 70% of the injected glargine is metabolised into M1 (M2 was not determined in this study) within half an hour; carboxypeptidates present in serum are likely to be involved in this process [131]. Bolli et al. and Lucidi et al. and Varewijck et al. concluded that in patients almost all glargine is converted into M1 with no significant conversion into M2 [83] [140] [141]. In our *in vitro* assay the majority of glargine appeared to be converted into M2 (60%) and a smaller part in M1 (40%). This difference in study outcome with regard to levels of metabolites could in part be explained by differences in enzyme levels in the serum. Regardless, it is clear that glargine metabolism plays an important role *in vivo*, and it is therefore essential to clarify which specific enzymes are involved in this process and to what extent this process differs from person to person.

To further substantiate our *in vitro* findings, we studied glargine metabolism *in vivo* in the mouse. Similar to the human situation glargine was rapidly metabolised. M1 appeared to be the predominant form and could be detected at high concentrations after glargine injections.

A subsequent Western blot analysis indicated that one hour after glargine injections activation of mitogenic pathways in the mammary glands is detectable, but there is no sustained activation of these pathways. These *in vivo* results underline that the *in vitro* results are also relevant for

the *in vivo* situation. The consequences for carcinogenic outcome of these brief pulses of mitogenic signalling caused by glargine treatment needs further evaluation.

In conclusion, we have developed a unique cell-based assay that can be used to systematically screen insulin analogues for their mitogenic potential in relation to activation of INSR or IGF1R. Using this assay it was shown that insulin glargine has an increased mitogenic activity comparable to that of AspB10, that acts mainly via the IGF1R. The physiological relevance of this *in vitro* finding to insulin glargine patients is yet unknown, since the injected glargine is rapidly metabolised *in vivo* into low mitogenic but metabolically active compounds.

Declaration of interest

There is no duality of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

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Author contributions

JWvdL, BvdW, CLES and BtB were responsible for the study concept and design. BtB, NK and EK for acquisition of data. BtB, BvdW, JWvdL and CLES analyzed and interpret data. BtB wrote the manuscript. BvdW, JWvdL, EK and CLES reviewed and edited the manuscript. All authors approved the final version of the manuscript.

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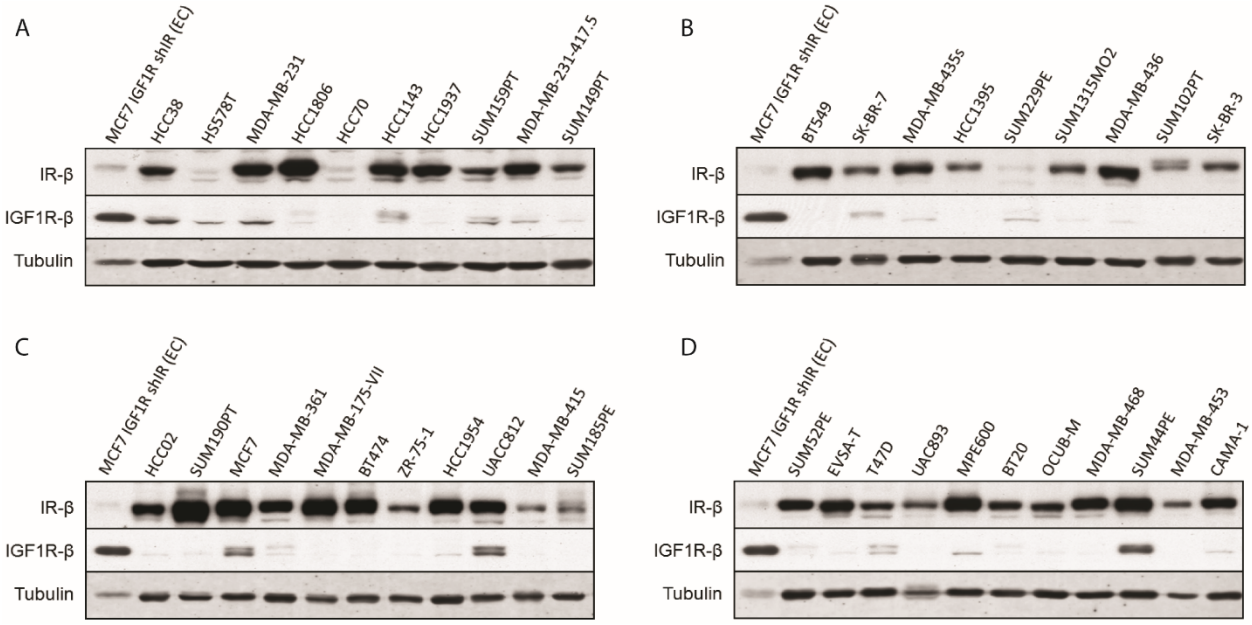
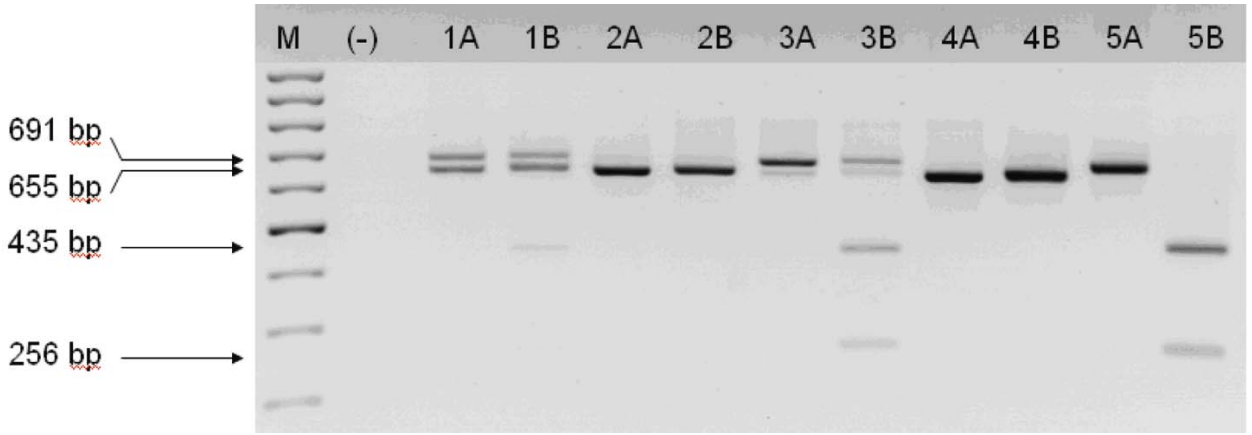
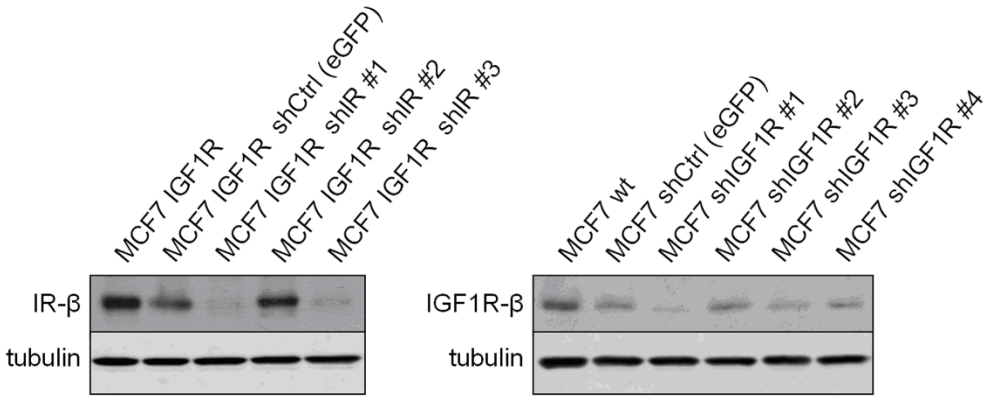
Supplementary Figures



Supplemental figure 1. Western blot analysis to determine the IR and IGF1R levels in 41 human breast cancer cell lines. a) and b) represent triple negative breast cancer cell lines. c) Luminal like cell lines and d) epithelial-like and "round" like cell lines. The MCF7 IGF1R shIR cell line was included as an endogenous control.



Supplemental figure 2. Restriction enzyme analysis with AVRII on Insulin receptor PCR fragment to check isoform type in MCF-7 wt, MCF-7 IRA and MCF-7 IRB. M=100bp marker. (-) represents a negative control (PCR mix) 1=MCF-7 wt PCR fragment, 2= MCF-7 IRA, 3=MCF-7 IRB, 4=pNTK2-IRA and 5=pBABE-IRB PCR fragment (A=uncut, B=cut with AVRII restriction enzyme). The 691 bp band represents the IRB PCR fragment. The 655 band represents the IRA fragment. AVRII will cut the IRB band into two smaller fragments of 435 and 256 bp.



Supplemental figure 3. Western blot analysis to determine knockdown efficiency of several shRNA constructs. shIR #1; TRCN0000000380, #2; TRCN0000000381, #3; TRCN0000000382. shIGF1R #1; TRCN0000000424, #2; TRCN0000000425, #3; TRCN0000000426, #4; TRCN0000039673.

Chapter 4

Alternative signaling network activation through different insulin receptor family members caused by pro-mitogenic antidiabetic insulin analogues in human mammary epithelial cells

Highlights

- IGF1 and AspB10 induce a very strong transcriptomic response, especially 1hr after stimulation
- A mitogenic gene expression signature could be defined that was validated with different models and showed a high predictive potential as compared to functional assays
- AspB10 and glargine are the only insulin analogues with an increased mitogenic potential compared to regular insulin as determined with this transcriptomic analysis

This chapter has been submitted as:

B. ter Braak, S. Wink, E. Koedoot, C. Pont, C.L.E. Siezen, J.W. van der Laan, B. van de Water

Alternative signalling network activation through different insulin receptor family members caused by pro-mitogenic antidiabetic insulin analogues in human mammary epithelial cells

Breast Cancer Research, resubmitted 9-04-2015

◀ IN THE PICTURE

Immunofluorescent microscopy set up. This type of microscopes is often used to detect fluorescent light emitted by your protein of interest. In this way a high resolution and magnified picture can be created so that specific cell features can be studied. By taking pictures over time, the migratory behaviour of certain cells under specific conditions can be studied.

◀ IN BEELD

immunofluorescentie microscoop opstelling. Dit type microscoop wordt vaak gebruikt om fluorescerend licht, uitgestoten door een te onderzoeken eiwit, te detecteren. Op deze manier kunnen hoge resolutie en vergrote plaatjes worden gecreëerd. Door op verschillende tijdpunten foto's te nemen, kan het migrerende gedrag van cellen onder bepaalde condities worden bestudeerd.

Abstract

Insulin analogues are designed to have improved pharmacokinetic parameters compared to regular human insulin. This provides a sustained control of blood glucose levels in diabetic patients. All novel insulin analogues are tested for their mitogenic side effects, however these assays do not take into account the molecular mode-of-action of different insulin analogues. Insulin analogues can bind the insulin receptor and the insulin-like growth factor-1 receptor with different affinities and consequently will activate different downstream signaling pathways. Here we used a panel of MCF7 human breast cancer cell lines that selectively express either one of the isoforms of the INSR or the IGF1R. We applied a transcriptomics approach to assess the differential transcriptional programs activated in these cells by either insulin, IGF1 or X10 treatment. Based on the differential expressed genes between insulin versus IGF1 and X10 treatment we retrieved a mitogenic classifier gene set. Validation by RT-Q-PCR confirmed the robustness of this gene set. The translational potential of these mitogenic classifier genes was examined in primary human mammary cells and in mammary gland tissue of mice in an *in vivo* model. The predictive power of the classifier genes was evaluated by testing all commercial insulin analogues in the *in vitro* model and defined X10 and glargine as the most potent mitogenic insulin analogues. We propose that these mitogenic classifier genes can be used to test the mitogenic potential of novel insulin analogues as well as other alternative molecules with an anticipated affinity for the IGF1R.

Keywords: insulin analogues, transcriptomics, IGF1R and INSR pathway, mitogenic classifier, breast cancer

Introduction

Diabetes mellitus is the most common endocrine disease with over 380 million patients in 2013, worldwide [142]. A common treatment for both type-1 and type-2 diabetics is the use of insulin analogues, which are insulin-like molecules with altered pharmacokinetic parameters so that they are either absorbed more rapidly or slower compared to regular insulin after injection. A combinational treatment with these short and long-acting insulin analogues provides the patient with normal blood glucose levels. These insulin analogues have been used for several decades, but recently some epidemiological studies found a correlation between the use of some of these compounds and the cancer occurrence, especially breast cancer [16] [76, 143] [144]. On the contrary, other epidemiological studies could not confirm these results and suggested that confounding factors (e.g. hyperinsulinemia and age of patients) might have caused this effect [64] [18] [19, 145, 146] [77]. There are two main hypotheses by which insulin analogues might increase the risk of cancer [147]. Firstly, the changes to the molecular structure of insulin affect the binding properties towards different receptors (e.g. insulin receptor-A (IRA) [134] or insulin-like growth factor-1 receptor (IGF1R) [148]). As a consequence these insulin analogues have an

increased mitogenic potential. In this scenario the insulin analogues could act either as a tumor initiator by transforming benign or (pre-) neoplastic cells which often express increased levels of IRA and IGF1R [136], or as a tumor promoter by stimulating the increased growth potential of these cells. Secondly, insulin analogues might induce mutagenic action either directly or indirectly as a statistical consequence of the increased mitogenic potential. However, evidence for an indirect enhanced mutagenic effect due to insulin analogue treatment has never been observed and therefore the first hypothesis is the most plausible scenario. As indicated before some insulin analogues have an increased binding potential towards the IGF1R [114] and/or a prolonged occupancy time for the IRA [149]. A simple evaluation of this effect has been the proliferative potential of insulin analogues, but the obtained results strongly depend on the used cell model and experimental procedures (reviewed in Bronsveld et al.) (Bronsveld, 2015 manuscript submitted) and are excluding the systematic evaluation of the actual role of the different insulin receptor families. We have developed a panel of MCF7 cell lines that express selectively either the IRA, IRB or IGF1R [46], which now allows us to differentiate the effect of individual insulin analogues on cellular signaling more precisely.

The downstream signaling of IRA and IGF1R is a complex diverse network leading to the activation of a diverse set of downstream cell signaling cascades and various transcription factors. The difference in activating either INSR or IGF1R signaling ultimately defines the cell biological outcome, roughly metabolic control versus pro-mitogenic signaling respectively. The diversity of signaling events can be mapped using proteome-wide phospho-proteomics analysis [46]. Alternatively genome-wide transcriptomics may more broadly define the different signaling networks that are activated by either INSR or IGF1R. For the safety evaluation of novel chemical entities transcriptomics-based profiling is often used to correctly classify the potential toxic properties [150] [151]. Given the differential activation of INSR and IGF1R by some insulin analogues, we anticipate that an IGF1R activation gene expression signature would be advantageous to define the mode-of-action of highly mitogenic insulin analogues. Therefore, in this study we used our MCF7 human breast cancer cell lines that differentially express the different insulin receptor family members [46]. We used transcriptomics to define gene sets involved in insulin analogue induced mitogenic signaling. These genes are candidate mitogenic classifiers to predict the mitogenic potential of newly developed insulin analogues or growth factors in general that act on the IGF1R.

Methods

Primary cell isolation, Cell line generation and cell culturing

Cell lines based on the human breast cancer MCF7 cell lines, which predominantly express the IRA, IRB or IGF1R have been described previously [46]. All MCF7 derivatives were cultured in

RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin (Invitrogen).

Primary human mammary cells have been isolated from cryopreserved biopsies of two individuals as described previously [152]. The two biopsies were obtained from two female patients who had undergone breast cancer-related surgery at the Leiden University Medical Center (LUMC). Procedures were followed according to the Dutch Medical Treatment Act and the study protocol was compliant with "the Code of proper secondary use of human tissue in the Netherlands" issued by the Dutch Federation of Medical Scientific Societies and approved by the Medical Ethical committee of the LUMC (P10.226). Specimens were coded anonymously in a way that they were not traceable back to the patient by laboratory workers. As much as possible fat tissue was removed from the human mammary biopsies, thereafter they were cut into 8 mm^3 pieces, which were then dried and attached to the culture flask for 30 minutes. 20% FBS containing DMEM-F12 medium (GIBCO/Invitrogen, Breda, the Netherlands) was gently added and refreshed every 5 days. Around the edges of the tissue, cells (mainly fibroblasts) started growing and after 3 weeks the culture flask was confluent with cells. The fraction of epithelial cells was enriched by multiple short trypsinisation steps in which part of the fibroblasts were removed. For 2 more passages the cells were cultured in HuMEC Ready Medium (GIBCO/Invitrogen). After this step the primary mammary cells were cultured in DMEM-F12 medium supplemented with 10% FBS and 100 U/mL penicillin-streptomycin (Invitrogen).

Insulin, insulin analogues and IGF1 in vitro stimulation

Prior to compound stimulation the cells were starved in 5% charcoal/dextran-stripped fetal bovine serum (CDFBS) containing medium. Stimulations included: insulin NPH (Insuman Basal, Sanofi Aventis), insulin glargine (Lantus, Sanofi Aventis), M1 (metabolite of glargine, Sanofi Aventis), M2 (metabolite of glargine, Sanofi Aventis), glulisine (Apidra, Sanofi Aventis), lispro (Humalog, Elly Lilly), insulin X10 (not marketed, Novo Nordisk), aspart (B28Asp, Novo Nordisk), detemir (Levemir, Novo Nordisk) and IGF1 (Increlex, Ipsen). All insulin analogues were dissolved in their original vehicle solutions [46]. For the *in vitro* experiments 1000x stock concentrations were prepared. Except for the first exposure experiment (Figure 1C) in which a dose response of 10, 33 and 100 nM was used, all exposures were performed with a concentration of 10 nM.

siRNA transfection

A transient transfection method with siRNA Smartpool mix (Dharmacon Technologies, Thermo Scientific, Lafayette, CO, USA) was used to test the effect of individual gene on cell proliferation. For this 10,000 MCF7 cells per well were seeded in 96-well plates in complete growth medium. 24 hours after seeding, 50 nM smartpool siRNA mix was delivered to the cells using a standard transfection method with DharmaFECT 4 transfection reagent (Dharmacon Technologies)

according to the company's instructions. 24 hours after transfection, the small interfering RNA (siRNA) transfection mixture was replaced with 5% CDFBS starvation medium for drug treatment and sulphorhodamine B (SRB) proliferation assay.

Sulforhodamine B colorimetric assay determining cell proliferation

A SRB assay was used to measure the total amount of protein as a measure for cell proliferation. Transfected and drug-treated cells in 96-wells plates were fixed with 30 µl 50% trichloroacetic acid directly added to 100 µl of assay medium per well for 1 hour at 4°C on a shaker, washed five times with distilled water and air-dried. Fixed cells were stained with 60 µl of 0.4% SRB (dissolved in 1% acetic acid) at room temperature on a shaker for 30 minutes. After the SRB protein binding, the plates were washed five times with 1% acetic acid to remove unbound dye and air-dried between the washing steps. Next, the protein-bound SRB in each well was solubilized in 200 µl 10 mM unbuffered Tris solution (pH>10) for 10 minutes on a plate shaker. Absorbance was measured at 530 nm with a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany).

Western blotting

Western blotting was used to determine the knockdown efficiency of the siRNA transfection. To prepare cell lysates for Western blot analysis, cells were washed two times with ice-cold PBS and lysed with 1x SPB with 1:20 β-mercaptoethanol. Samples were boiled at 95°C for 5 minutes and stored at -20°C. Before loading, samples were denatured at 95°C for 5 minutes. 20 µl (about 30 ug) protein solution per lane was separated by SDS-polyacrlyamide gel electrophoresis on a 7.5% acrylamide gel and electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Prior to primary antibody probe, membranes were blocked for 1 hour at room temperature with 5% bovine serum albumin (BSA) or 5% milk in Tris-bufferd saline Tween 20 (TBST) buffer (100mM Tris, pH 7.4, 500mM NaCl, 0.05% Tween 20). ERK, AKT, PTEN and tubulin antibodies were probed in 1% BSA-TBST buffer, whereas IGF1Rβ antibodies were probed in 1% milk-TBST buffer. HRP-conjugated secondary antibody incubation was performed in 1% BSA-TBST or 1% milk-TBST buffer, corresponding to the primary antibodies used. Protein bands were visualized by using the ECL (Amersham) method, after which the membrane was scanned by using a Typhoon 9400 imager (GE Healthcare). Anti-phospho-Akt (Ser473) and anti-phospho-Erk (Thr202, Tyr204) have been purchased from Cell signaling Technology, MA, USA). For a detailed description of the methods and origin of the antibodies we refer to our prior publications [46] [153].

Microarray studies

For the microarray the cells were seeded at a confluence of 60% in 6 cm plates, starved for 2 days in 5% CDFBS containing medium, followed by 1 hr or 6 hrs compound stimulation (10 nM) in serum free medium. Small and large RNA was isolated and purified using NucleoSpin® miRNA isolation kit (Machery Nagel, Düren, Germany) according to manufacturers' instructions. RNA quality and integrity were assessed by using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA). The Affymetrix 3' IVT Express Kit (Affymetrix, Santa Clara, CA, USA) was used to synthesize biotin-labeled cRNA, and this was hybridized to an Affymetrix HG-U133plus PM Array plate reader. Probe annotation was performed using the hgu133plus2.db package and probe mapping was performed with hgu133plus2cdf package installed using Bioconductor version 3.0. Probe-wise background correction (Robust Multi-array Average expression measure), between-array normalization (quantile normalization) and probe set summaries (median polish algorithm) were calculated with the RMA function of the Affymetrix package (Affy package version 1.38.1) [154]. The normalized data were statistically analysed for differential gene expression using a linear model with coefficients for each experimental group [155]. A contrast analysis was applied to compare each exposure with the corresponding vehicle control. For hypothesis testing the moderate t-statistical by empirial Bayes moderation was used followed by an implementation of the multiple testing correction of Benjamini and Hochberg [156] using the LIMMA package [157]. The microarray data is publically available at the Gene Expression Omnibus (GEO) database via accession number GSE65398.

RT-Q-PCR

For the qPCR analysis, messenger RNA from MCF7 cells (80% confluent 6-well) or mammary glands (30 µg tissue) was isolated/purified using NucleoSpin® miRNA isolation kit (Machery Nagel, Düren, Germany). cDNA was made using the universal cDNA synthesis kit (Exiqon). qPCR was performed in triplicate using SYBR Green PCR (Applied Biosystems) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Primers targeting the mitogenic classifiers have been manually designed and are listed in Supplemental Table 1. qPCR data were collected and analysed using SDS2.3 software (Applied Biosystems). Relative gene expression was calculated after correction for β-actin expression using the $2^{-\Delta\Delta Q}$ method. Data are presented as fold change (or log2 fold change) compared to vehicle stimulation.

Animal experiments

40 female 12-week-old inbred FVB/NRj mice were obtained from Janvier, rodent research models, France. Housing and experiments were performed according to the Dutch guidelines for the care and use of laboratory animals (UL-DEC-14020). RM2 food SDS (technilab-BMI, Holland) and water were provided *ad libitum*. Animals received a single subcutaneous injection of 100 µl

compound/vehicle solution. The doses were chosen so that the glucose drop was constant among the different compounds (Supplemental Figure 3A) (glargine and insulin 100 nmol/kg, X10 1200 nmol/kg and IGF1 12.5 mg/kg). One or six hours after the injection the mice were sacrificed, blood was collected (mini collect, Greiner/Omnilabo), blood glucose levels were measured (Freestyle light, 70812-70, Abbott), the 3rd and 4th mammary glands were isolated and used for Western blot protein quantification and quantitative PCR respectively (see Ter Braak, 2014 and 2015) [46] [153]. For each condition (treatment/time point) 4 mice were included.

Statistical analysis

For the statistical analysis of the microarray data, R (version 3.1) software was used. Rest of the analysis was performed with Graphpad Prism version 4.00. Student's t-tests were used to determine significance between conditions. P-values lower than 0.05 were considered to be significant. In all graphs the error bars represent standard deviations.

Results

Mitogenic signaling is regulated via highly similar signaling cascades in the INSR and IGF1R signaling pathway

To better understand the involvement of the IRA, IRB and IGF1R pathways (Figure 1A) in the context of mitogenic signaling of insulin analogues, we used our previously described human MCF7 breast cancer cell lines that express either IRA, IRB or IGF1R [46]. Exposure of these individual cells to the pro-mitogenic insulin analogue X10 that activates both the INSR and the IGF1R, resulted in intact downstream signaling cascades in all three cell lines, indicating functionality of the receptors (Figure 1B). As a next step we wanted to ensure that the IRA, IRB and IGF1R are not entirely different regarding their key intracellular proliferative signalling pathways. For this purpose, we tested the proliferative potential of the cells after knockdown of several key signaling molecules in these pathways (Figure 1A). As a first step, we optimized the knockdown efficiency using IGF1R and ERK1/2 as controls. The knockdown efficiency of IGF1R was almost 100% and constant over five days of culturing; the knockdown efficiency of ERK1 and ERK2 was around 50% after day 1 till 95% at day 5 (Figure 1C), we assume that the knockdown efficiency is constant for other targets but obviously it was practically not feasible to test them all in this manuscript. To assess the proliferative and anti-apoptotic effects of these knockdowns, we used the SRB proliferation assay. After knockdown of ERK1/2 and IGF1R, the amount of cells after five days of culturing was significantly decreased with 25% (Figure 1C), indicating that the SRB proliferation assay is a sensitive assay to pick up any anti-proliferative effects.

Next, we determined the effect on proliferation of ten individual signaling molecules that are key in the INSR/IGF1R signaling pathway (INSR, IGF1R, GRB2, RAF1, ERK2, IRS1, PI3KCA, PTEN, AKT2, GSK3B) in untreated IRA, IRB and IGF1R MCF7 cell lines (Figure 1D). As expected, the INSR

knockdown only significantly affected the proliferative behaviour in the IRA and IRB cell line. Similarly, the siIGF1R only significantly reduced the proliferative behaviour in the MCF7 IGF1R cell line. Transient knockdown of downstream targets in the MAPK signaling cascade (GRB2, RAF1 and ERK2) all had a significant inhibiting effect on proliferation. Also knockdown of targets in the PI3K signaling cascade (IRS1, PI3KCA and AKT2) had a significant reduced proliferative effect in all cell lines. It has to be noted that different members of the AKT family have redundant functions and can therefore take over the loss of function of the silenced member. We expect that a knockdown of all three members of AKT would lead to an even stronger effect on cell proliferation [158]. As anticipated, a knockdown of PTEN increased the proliferative potential in these cells, since PTEN acts as a tumor suppressor through dephosphorylation of phosphoinositide-3 phosphate, thereby negatively regulating PI3K signaling. Also GSK3B knockdown showed an increase in proliferation. The anti-apoptotic effects of GSK3B are likely to cause this effect which was only detected in the IGF1R overexpressing cell line. Finally, the proliferative potential of non-stimulated MCF7 IGF1R cells treated with different siRNAs was compared to insulin and IGF1 treatment conditions (Figure 1E). The effect of the stimulation itself was clearly detectable as the SRB absorbance increased from 1.04 (untreated, upper graph) to 1.81 (insulin treated, middle graph) to 2.61 (IGF1 treated, lower graph) in the mock condition. Furthermore the effects of the different siRNA knockdowns on proliferation became more prominent in the stimulation conditions. Interestingly, in the insulin stimulated condition the siINSR significantly affected proliferation in the IGF1R cell line, suggesting that the low levels of INSR in this cell line (Figure 1B) are involved in proliferation once stimulated with high levels (10 nM) of insulin.

It could be argued that the effects described above are not (solely) due to pro-mitogenic effects, since the INSR/IGF1R signalling pathway can also induce anti-apoptotic effects (See figure 1A). These anti-apoptotic effects could also lead to more cells and thus a higher SRB assay read out. To investigate this we determined the apoptotic fraction with a FACS analysis upon stimulation with the different growth factors (insulin, glargine, X10, IGF1) at 0, 10 and 100 nM. As expected, we found a slightly, but dose dependent, higher fraction of apoptotic cells in the growth factor stimulated cells (~6%) versus the unstimulated (~4%) (data not shown). Since this is such a small difference we assume that the anti-apoptotic effects play a minor role compared to the pro-mitogenic effects in the growth factor stimulation experiments.

In conclusion, these combined data indicates that the core signaling pathways involved in cell proliferation are similar in the entire MCF7 INSR family cell panel, allowing us to use this panel to further unravel the signaling events that can differentiate between INSR versus IGF1R acting insulin analogues.

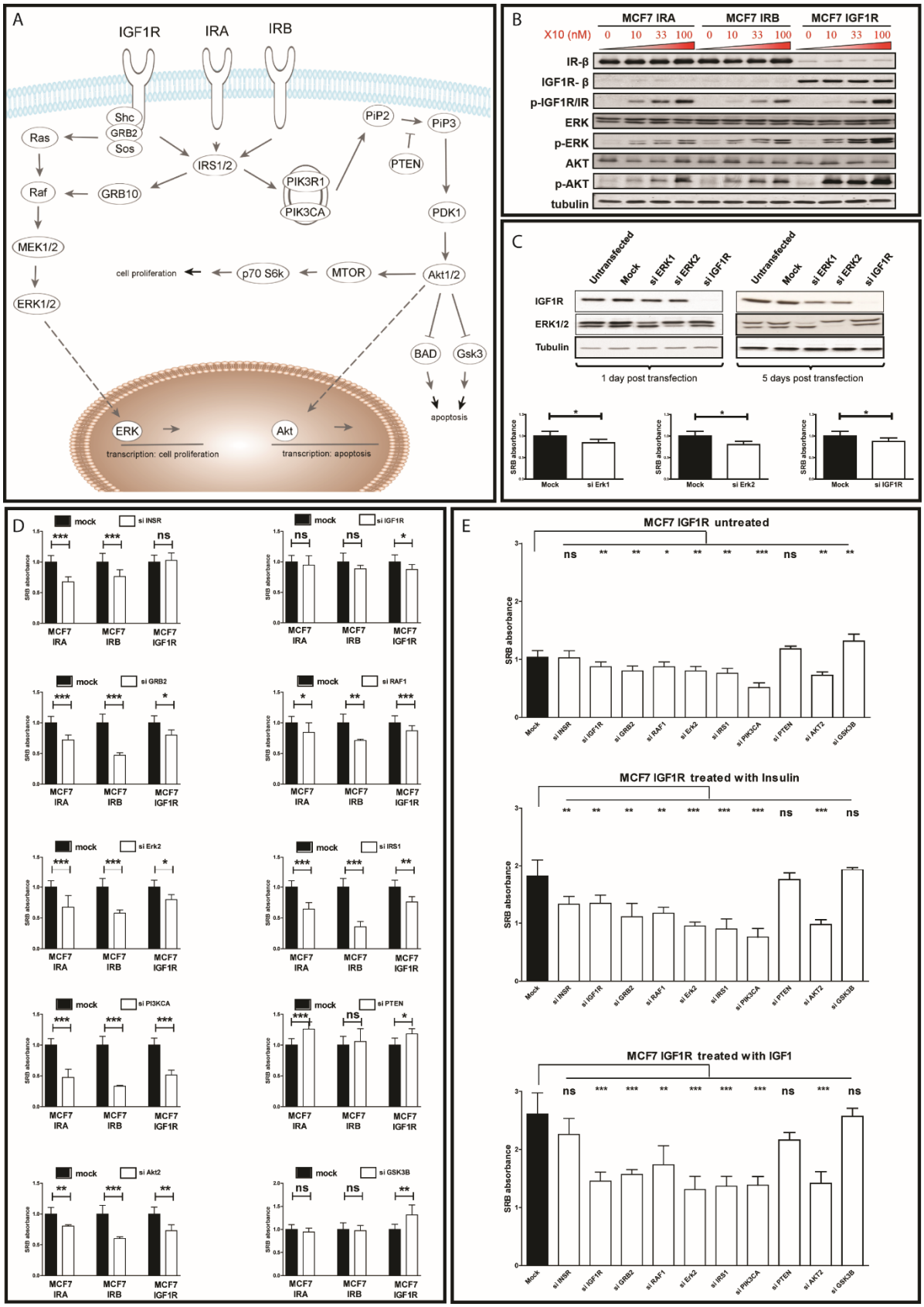


Figure 1. Knockdown of signaling components critical in the INSR and IGF1R pathway reveals common canonical core in IRA, IRB and IGF1R induced proliferation signaling. A) The canonical INSR and IGF1R signaling pathway with the focus on proliferative and apoptotic biological outcomes. B) Western blot analysis of the cell line panel, based on the human breast cancer cell line MCF7 with stable retroviral overexpression (IRA, IRB and IGF1R) in combination with stable a short hairpin knockdown (INSR and IGF1R). Cells have been treated with 0, 10, 33 or 100 nM of insulin

X10 for 30 min. Downstream signaling pathway activation of the receptors is intact as is indicated by the dose-dependent activation of p-ERK/p-AKT. C) Western blot analysis of siRNA transfection efficiency in the MCF7 IGF1R cell line, 1 day and 5 days post transfection and the effect of the knockdown on proliferation measured with the SRB proliferation assay. D) The effect of transient knockdown of ten important signaling molecules (INSR, IGF1R, GRB2, RAF1, ERK2, IRS1, PIK3CA, PTEN, AKT2 and GSK3B) in the INSR and IGF1R signaling pathways on SRB proliferation measured in the different MCF7 derivatives (MCF7 IRA, MCF7 IRB and MCF7 IGF1R). E) The effect of treatment and knockdown of key signaling molecules in INSR and IGF1R signaling on SRB proliferation measured in MCF7 IGF1R. (* p<0.05, ** p<0.01, *** p<0.001)

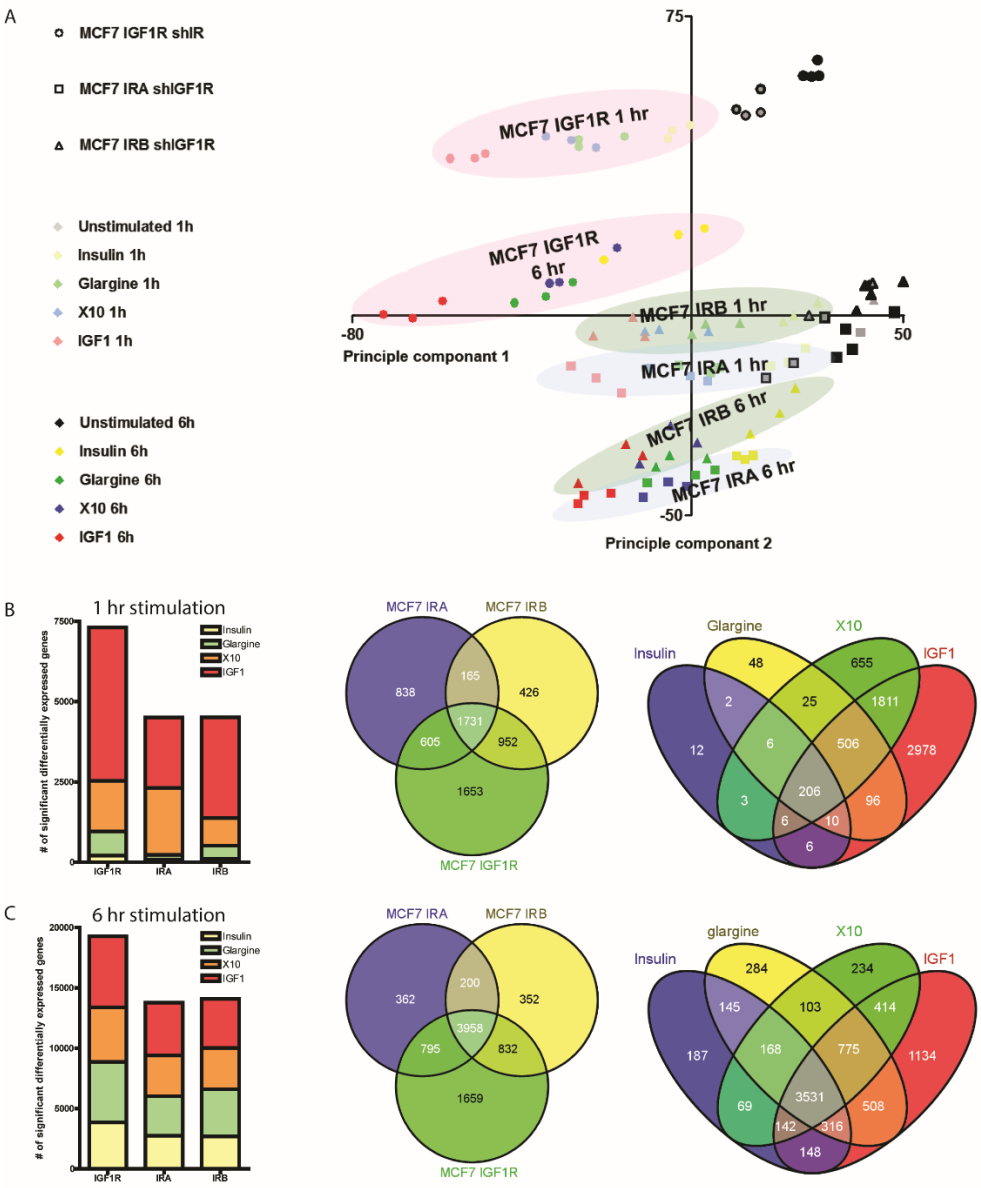


Figure 2. Experimental set-up of microarray experiment. A) 2D Principle Component Analysis plot of the microarray gene expression data. A clear separation of the different treatments (indicated by the different colors of the dots), cell lines (different shapes) and time points (light; 1 hr vs dark; 6 hrs) could be observed. The triplicates or quadruplicates are as expected very close to each other. B) Number of significantly differentially expressed genes between the different cell lines at T=1 hr. The first Venn diagram shows overlap of the significant differentially expressed genes (DEGs) per cell line at T=1 hr. The second Venn diagram shows overlap of the significant DEGs per treatment at T=1. C) As in B) but then for T=6 hrs.

Insulin analogues trigger different transcriptomes in the different cell lines.

To detect the differences in gene expression levels between the different cell lines, we next performed a microarray experiment using the same cell line panel (Figure 1B). We included 5 different stimulation conditions (vehicle, insulin, glargine, X10 and IGF1 stimulation). This allowed the comparison of the transcriptomes of the different treatments. We also included two different time points, thus enabling observation of possible time dynamics. Using a principle component analysis (PCA) a clear separation of the different treatments and cell lines conditions was observed (Figure 2A). The PCA indicated that the transcriptome after IGF1 treatment was most different from vehicle stimulation. Glargine and X10 treatments cluster together and insulin treatment is closest to the vehicle treated situation. Triplicate (or quadruplicate for the vehicle) samples from independent biological experiments were close to each other, indicating a strong robustness of the assay.

We determined the significantly differentially expressed genes (DEGs) per condition (1 hour stimulation, Figure 2B; 6 hours stimulation, Figure 2C). Most DEGs were observed in the IGF1R cell line, which is consistent with the strongest separation of this cell line in the PCA analysis (Figure 2A). We combined all DEGs per cell line and determined the overlap from the different treatments. There was a 43 % overlap in the DEGs between the different cell lines. We also compared the treatment specific responses independent of the cell line (right Venn diagram Figure 2B/C). Again, IGF1 treatment has the biggest impact on the transcriptome, and the highest overlap with the X10 and glargine treatment. The total number of DEGs 6 hour after stimulation (Figure 2C) is considerably larger compared to the 1 hr stimulation (Figure 2B). After 6 hours of stimulation there was a large overlap of DEGs among the different cell lines as well as treatments (3531), suggesting that at this time point more general mechanisms were activated that are similar for the different treatment conditions. In Supplemental Figure 1 the Venn diagrams of all the different conditions are presented. A noteworthy finding is the very high number of DEGs in the X10 stimulation via the IRA at T=1 hr.

Differential pathway activation by the various insulin analogues.

To further understand the biological pathways up regulated by these different compounds we performed an Ingenuity Pathway Analysis (IPA), focussing on the MCF7 IGF1R cell line using both time points (Venn diagram Figure 3A). A *mitogenic cluster* was defined that included all DEGs of IGF1 treatment only, or IGF treatment in combination with X10 and/or glargine treatment. We included glargine treatment in this cluster as glargine, like X10, is highly mitogenic in the absence of serum [46]. In a similar way a *metabolic cluster* was defined, including all DEGs of insulin treatment only, or insulin treatment in combination with glargine and/or X10 since all these compounds are known to have a strong metabolic effect *in vivo*. Using IPA we found 'ERK/MAPK' and 'p70S6K' signaling pathways significantly enriched in the *mitogenic cluster*, while the 'PI3K'

and 'Cell cycle control' pathways were not enriched. For the *metabolic cluster* the IPA results were the other way around. We also performed IPA analysis on the individual treatment DEG lists. 'Cell cycle control of chromosomal replication' was highly enriched after treatment with compounds with a high affinity for the INSR (insulin, glargine and X10). Other metabolic processes like glycogen degradation and D-myo-inositol-5-phosphate metabolism were also enriched in the DEGs of these insulin molecules. On the other hand PI3K/AKT, IGF1, p53 and ERK/MAPK signaling were more enriched for the insulin-like molecules that also have a strong affinity for the IGR1R.

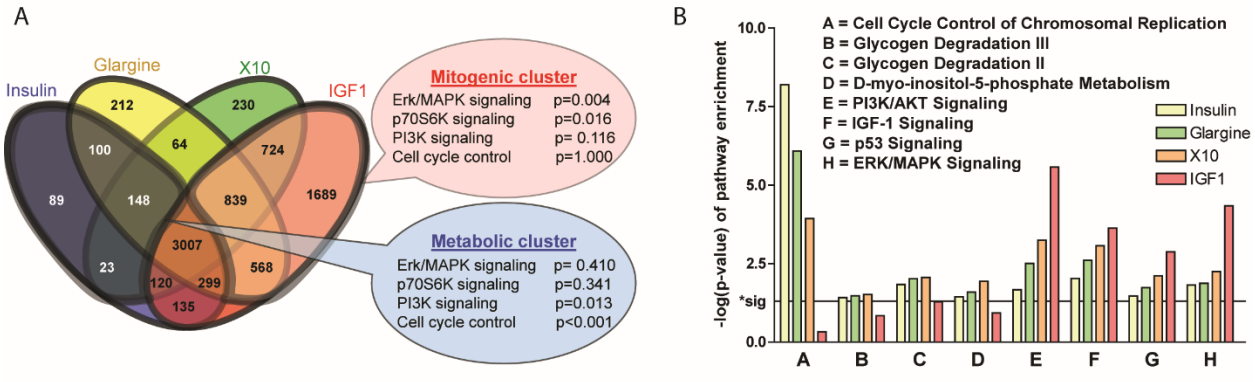


Figure 3. Pathway enrichment analysis of differentially expressed gene lists. A) Separate gene clusters were defined based on the Venn diagram of MCF7 IGF1R. The mitogenic gene cluster consists of all DEGs in IGF1 treatment alone and the combinations of IGF1 with glargine and/or X10 treatment. Similar a metabolic gene cluster was defined including the insulin specific DEGs with combinations of the other insulin analogues. An Ingenuity Pathway Analysis (IPA) was performed that revealed an enrichment of the ERK/MAPK and p70S6K signaling pathways in the mitogenic cluster whereas the PI3K ad cell cycle control signaling pathways were enriched in the metabolic cluster. B) An IPA analysis was performed on the DEGs of individual treatments including all cell lines and the different time points. As expected the metabolic signaling (A t/m D) was up regulated after stimulation with metabolic compounds (insulin, glargine and X10). IGF1 stimulation led to a very significant up regulation of PI3K/AKT, ERK/MAPK, IGF1 and p53 signaling. For insulin signaling these pathways were also enriched but less significant.

A classifier gene set predictive for the pro-mitogen action of insulin analogues.

To evaluate which genes drive the strong mitogenic responses of IGF1R signaling we performed a variance test with selected genes showing a strong up or down regulation after strong activation of the IGF1R. For this we selected IGF1 and X10 exposures and contrasted this with the weak mitogenic response inducer insulin. We excluded glargine for the selection. In total we selected the top 10 hits in both the 1 hr and 6 hrs hit lists (Figure 4A). Interestingly, many of these genes are known to play a role in mitogenic processes, including the early growth response (EGR) genes (all four EGR genes are in the top 20 gene list). Most of these genes have not directly been linked to the INSR or IGF1R signaling pathway so far. Next we validated these candidate genes using RT-Q-PCR with a separate independent set of samples. For 18 of these mitogenic classifier genes the RT-Q-PCR validation was successful and showed a highly similar trend for insulin, X10 and IGF1 conditions (Figure 4B). For ZIC4 and ZMYND8 the expression was probably too low since

no amplicon was detected even after 40 cycles. Finally, we evaluated the effect of glargine on the expression of these classifier genes. Importantly, the overall expression of the classifier genes after glargine treatment was more similar to X10 than insulin treatment.

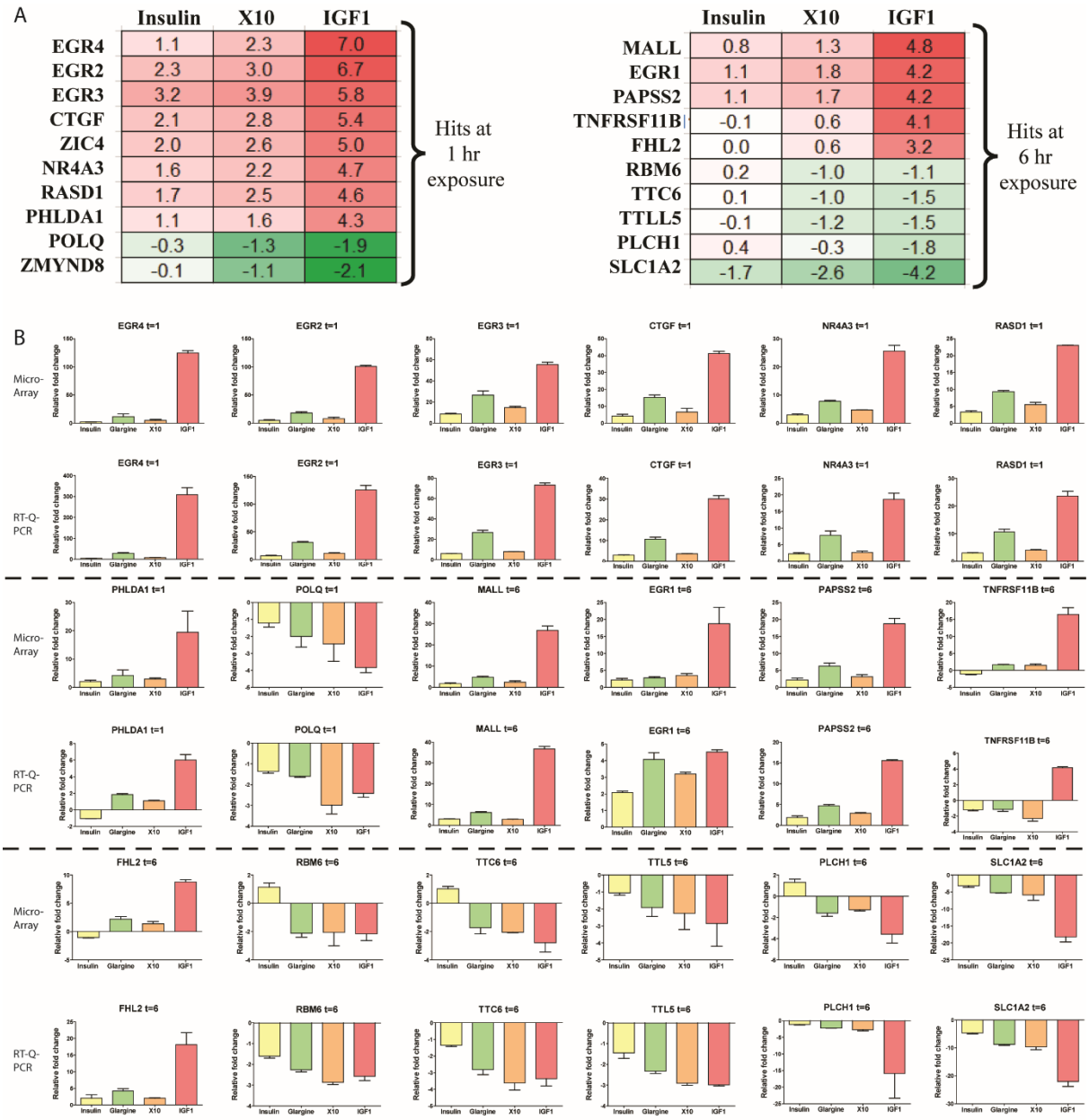
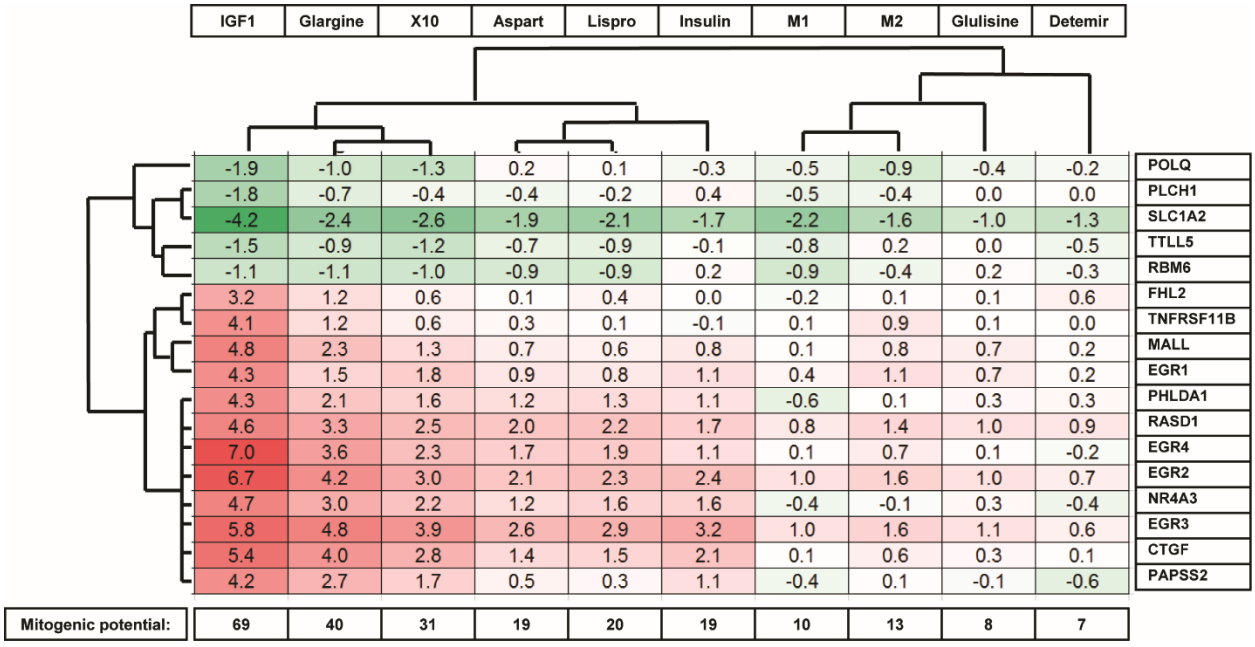


Figure 4. Twenty mitogenic classifier genes discriminating between insulin and X10/IGF1 signaling. A) Twenty genes (10 for each time point) were defined of which the gene expression was most significantly up or down-regulated in X10/IGF1 vs insulin in the MCF7 IGF1R cell line (based on a variance test), values are presented as log fold 2 changes. B) Validation of 18 of these mitogenic classifier genes was successful with RT-Q-PCR. A comparison is given of the microarray (top panel) vs RT-Q-PCR (lower panel) gene expression of the mitogenic classifiers. Expression is indicated as fold changes relative to unstimulated MCF7 IGF1R.

Validation of mitogenic classifiers through testing of commercially available insulin analogues.

We hypothesized that the expression of the mitogenic classifier genes could predict the mitogenic outcome of other insulin analogues. We performed an exposure experiment with

MCF7 IGF1R cells including all commercially available insulin analogues (glargine, aspart, lispro, glulisine, determir). Since glargine showed expression of the predictive genes (Figure 4B), and since glargine is rapidly metabolized into two metabolites (M1 and M2) in serum, we also included M1 and M2 in our study. A hierarchical clustering of the expression of all the tested classifier genes after stimulation with the different insulin analogues was performed (Figure 5). This resulted in the clustering of glargine with IGF1 and X10, while the glargine metabolites M1 and M2 clustered with other relatively non-mitogenic insulin analogues. We calculated a 'relative mitogenic potential' which was determined as the sum of the absolute values of log 2 fold changes of the expression of mitogenic classifiers of one compound treatment. As expected the 'relative mitogenic potential' was highest for IGF1 (69), followed by glargine (40) and X10 (31). The mitogenic potential of insulin (19) was very similar to that of aspart (19) and lispro (20). M1 (10), M2 (13), glulisine (8), and determir (7) showed a lower predicted mitogenic potential compared to regular insulin.



	IGF1	Glargine	X10	Aspart	Lispro	Insulin	M1	M2	Glulisine	Detemir	
	-1.9	-1.0	-1.3	0.2	0.1	-0.3	-0.5	-0.9	-0.4	-0.2	POLQ
	-1.8	-0.7	-0.4	-0.4	-0.2	0.4	-0.5	-0.4	0.0	0.0	PLCH1
	-4.2	-2.4	-2.6	-1.9	-2.1	-1.7	-2.2	-1.6	-1.0	-1.3	SLC1A2
	-1.5	-0.9	-1.2	-0.7	-0.9	-0.1	-0.8	0.2	0.0	-0.5	TTLL5
	-1.1	-1.1	-1.0	-0.9	-0.9	0.2	-0.9	-0.4	0.2	-0.3	RBM6
	3.2	1.2	0.6	0.1	0.4	0.0	-0.2	0.1	0.1	0.6	FHL2
	4.1	1.2	0.6	0.3	0.1	-0.1	0.1	0.9	0.1	0.0	TNFRSF11B
	4.8	2.3	1.3	0.7	0.6	0.8	0.1	0.8	0.7	0.2	MALL
	4.3	1.5	1.8	0.9	0.8	1.1	0.4	1.1	0.7	0.2	EGR1
	4.3	2.1	1.6	1.2	1.3	1.1	-0.6	0.1	0.3	0.3	PHLDA1
	4.6	3.3	2.5	2.0	2.2	1.7	0.8	1.4	1.0	0.9	RASD1
	7.0	3.6	2.3	1.7	1.9	1.1	0.1	0.7	0.1	-0.2	EGR4
	6.7	4.2	3.0	2.1	2.3	2.4	1.0	1.6	1.0	0.7	EGR2
	4.7	3.0	2.2	1.2	1.6	1.6	-0.4	-0.1	0.3	-0.4	NR4A3
	5.8	4.8	3.9	2.6	2.9	3.2	1.0	1.6	1.1	0.6	EGR3
	5.4	4.0	2.8	1.4	1.5	2.1	0.1	0.6	0.3	0.1	CTGF
	4.2	2.7	1.7	0.5	0.3	1.1	-0.4	0.1	-0.1	-0.6	PAPSS2
Mitogenic potential:	69	40	31	19	20	19	10	13	8	7	

Figure 5. The relative mitogenic potential of various insulin analogues determined by the classifier gene expression. A) MCF7-IGF1R cells were exposed to IGF1, X10, glargine, insulin, aspart, lispro, M1, glulisine, M2 and detemir and the gene expression of the mitogenic classifier genes was measured with RT-Q-PCR. A hierarchical clustering of the log fold 2 changes (compared to vehicle stimulation) is shown. The mitogenic potential score is the absolute sum of the absolute values of log 2 fold changes of the mitogenic classifiers.

Validation of mitogenic classifier genes in vitro in primary human mammary gland cells and in vivo in mouse mammary glands.

To further validate the insulin analogue mitogenic classifier genes we tested additional in vitro and in vivo models. We first determined the robustness of the insulin analogue mitogenic classifier genes in primary cultured cells isolated from human mammary glands. These cells were

anticipated to be the main target for increased mitogenic signaling of insulin analogues in diabetic patients. Primary cells were isolated from two independent individuals and exposed to the different insulin-like molecules. The activation of the INSR/IGF1R pathway was validated by Western blotting (Supplemental Figure 2) and a clear activation of the INSR/IGF1R as well as the PI3K/AKT signaling pathway was observed. Next the gene expression levels of three mitogenic classifier genes that were up regulated (EGR4 and TNFRSF11B) or down regulated (SLC1A2) in MCF7 IGF1R cells were measured (Figure 6A).

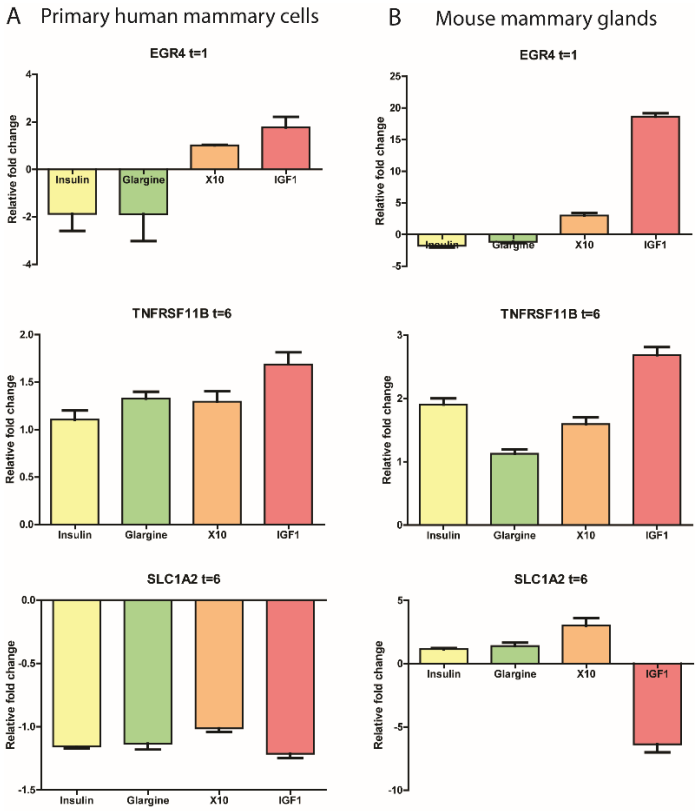


Figure 6. Validation of insulin analogue mitogenic classifier genes in primary human mammary cells and *in vivo* mouse mammary glands. A) Primary cells isolated from two different human mammary biopsies were exposed to insulin, glargine, X10 or IGF1 and the gene expression of EGR4, TNFRSF11B and SLC1A2 were determined by RT-Q-PCR. Values are presented as fold changes relative to vehicle treated cells. B) Wild type FVB mice (4 per condition) were subcutaneously injected with the above mentioned compounds, RNA from the mammary glands was isolated and the gene expression of EGR4, TNFRSF11B and SLC1A2 was measured by Q-RT-PCR and indicated as fold change relative gene expression of vehicle injected mice.

Although the fold change expression of these three genes in these primary human mammary cells was not as profound compared to the MCF7 IGF1R cells, in general the same direction of expression was observed. In addition, we investigated these three classifier genes in vivo in the mammary glands of mice treated with the different insulin analogues. In this experiment 40 wild type FVB mice received a subcutaneous injection of vehicle, insulin, glargine, X10 or IGF1. A very clear and constant drop in the glucose levels was observed 1 hour after the injections of insulin, glargine, X10 and IGF1, indicating that these compounds did induce the expected pharmacological response (Supplemental Figure 3A). The glucose levels returned to their normal levels (5 mmol/L), 6 hours after the injection. We then investigated the activation of the INSR and IGF1R (Supplemental Figure 3B and 3C). 1 hour after the insulin analogue injections a clear up regulation of p-AKT was observed, while after 6 hours the p-p70S6K levels were significantly ($p=0.0022$) up regulated. Also the insulin analogue mitogenic classifier genes showed a very clear

modulation after treatment (Figure 6B). Thus, EGR4 was even induced up to 18 times after IGF1 treatment and X10 also showed a clear up regulation of this candidate gene compared to no stimulation. Similarly after 6 hours treatment IGF1 induced TNFRSF11B and down regulated SLC1A2 levels. For these latter gene changes none of the other insulin-like molecules caused a significant change in gene expression. Gene expression in glargine conditions showed a similar trend as regular insulin, suggesting that glargine is rapidly metabolized into M1 and M2 in vivo, which are known to be compounds with a low pro-mitogenic signaling potential [46].

Discussion

It is well established that insulin has strong metabolic effects and in addition mild pro-mitogenic characteristics [148]. Small changes in the structure of insulin have improved the pharmacokinetic parameters so that the use of the insulin analogue is more convenient for diabetic patients. Yet, these small structural changes might also increase the binding affinity of insulin analogues towards the IGF1R and, consequently, increase the mitogenic potency of these molecules compared to regular insulin. Current *in vitro* systems that are used to determine mitogenic potential of insulin analogues are largely based on the proliferation capacity and do not take into account the molecular mechanisms of receptor signaling (Bronsveld 2015 manuscript submitted). In this study we used a transcriptomics approach to assess the preferential activation of pro-mitogenic signaling pathways by insulin analogues. We identified a subset of classifier genes that can be used to define the primary mode of action of insulin analogues. Moreover, we demonstrated that these classifier genes can be translated to primary human mammary cells as well as mouse mammary glands *in vivo*. These mechanism-based novel predictive genes are likely a more reliable method to classify the proliferative potency of insulin analogues that act preferably on the IGF1R.

For the safety profiling of insulin analogues this increased mitogenic potential is critical. Currently there is still a debate on the mechanism of such an increased mitogenic potential: on one hand the high binding affinity towards the IGF1R, while on the other hand a prolonged occupancy time towards the IRA is suggested causative [149]. In our current study we have been in the unique situation to evaluate these mechanisms in our MCF7 cell line panel. We found 40% more differentially expressed genes in the MCF7 IGF1R cell line after X10 and glargine stimulation compared to the MCF7 IRA cell line. These results suggest that the IGF1R is the main receptor that is mediating downstream pro-mitogenic signaling after insulin analogue stimulation. This is in line with our previous study in which we tested the proliferative potency of nine insulin-like molecules using the same MCF7 cell line panel and found that X10 and glargine induce proliferation more profoundly in the MCF7 IGF1R cells than the MCF7 IRA cells [46]. For this reason we based the further mitogenic classifier analysis on the MCF7 IGF1R cell line.

For the mitogenic classifier hit selection a training set was based on microarray gene expression of three compounds, in which insulin served as the reference compound with a low mitogenic

potency. X10 and IGF1 served as two insulin-like molecules with a strong mitogenic potential. This resulted in a total of twenty genes either up or down regulated at 1 or 6 hrs after IGF1 and X10 treatment that most strongly differed from the insulin effect. Many of these genes have been associated with mitogenic signaling but so far not directly linked to INSR/IGF1R signaling. Strikingly various early growth response genes were identified: EGR1 [159], EGR2 [160], EGR3 [161], EGR4 [162] which are all well known to promote proliferation, survival and/or invasion pathways. MALL, FHL2 [163], PHLDA [164], NR4A3 [165] and CTGF [166] are known oncogenic factors and its gene expression is negatively associated with tumor-free survival and/or. Interestingly, genes (POLQ and RBM6) that were down regulated after X10/IGF1 stimulation have been linked to pro-apoptotic and anti-proliferative effects [167] [168].

Glargine is the most frequently prescribed anti-diabetic insulin analogue. There are conflicting conclusions regarding the intrinsic mitogenic potential of insulin glargine [127] [169]. We purposely excluded our glargine transcriptome analysis from the training set to identify candidate predictive classifier genes for pro-mitogenic signaling by insulin analogues. This allowed us to unravel the potency of glargine as a pro-mitogenic insulin analogue. Interestingly, the mitogenic potential of glargine was even higher compared to insulin X10 (Figure 5). This is in full agreement with the kinase activation measurement of INSR/IGF1R pathway components in our previous study [46]. In diabetic patients glargine is rapidly processed by enzymes in the serum into two metabolically active compounds, M1 and M2, in which M1 is most prominent metabolite [83]. Therefore we also determined the mitogenic potential of M1 and M2 based on the gene expression profiles of the mitogenic classifier genes and we observed that both metabolites have a mitogenic score that is even lower than insulin. This is in agreement with previous studies in which the mitogenic potential was based on IGF1R binding affinity, kinase activation or proliferation assays [114] [46].

Two other studies also performed a mitogenic assessment of a panel of insulin analogues. These studies included a proliferation assay (Kurtzhals, Schaffer et al. 2000) and an IGF1R affinity evaluation [114]. We systematically compared our mitogenic classifier gene score with these two independent functional read outs (Figure 7). There was a striking correlation between our classifier scoring (based on Figure 5) and both the proliferation and IGF1R affinity. These combined data sets demonstrate that IGF1, X10 and glargine have a higher mitogenic potential compared to insulin, which is associated with a high affinity for IGF1R. Aspart and lispro have a mitogenic potency similar to each other. Determir and the two metabolites of glargine (M1 and M2) have a lower mitogenic index compared to regular insulin, associated with a low affinity for the IGF1R.

Some epidemiological studies suggest a correlation between insulin glargine use and breast cancer occurrence in the diabetic patients [76]. Since glargine might promote proliferation of mammary epithelial cells *in vivo*, we wanted to test whether expression of some of our classifier genes could be translated from MCF7 cells to primary human mammary cells. We could confirm a similar gene

expression trend in primary human mammary cells after stimulation with insulin, glargine, X10 and IGF1 as in MCF7. Yet, the gene expression fold changes in the primary human mammary cells were far lower compared to the MCF7 IGF1R cells. The reason for this can be partly due to the isolation procedure which did not result in a pure population of mammary epithelial cells. Overall, the effect of glargine in the primary human mammary cells was not as profound as for IGF1 and X10.

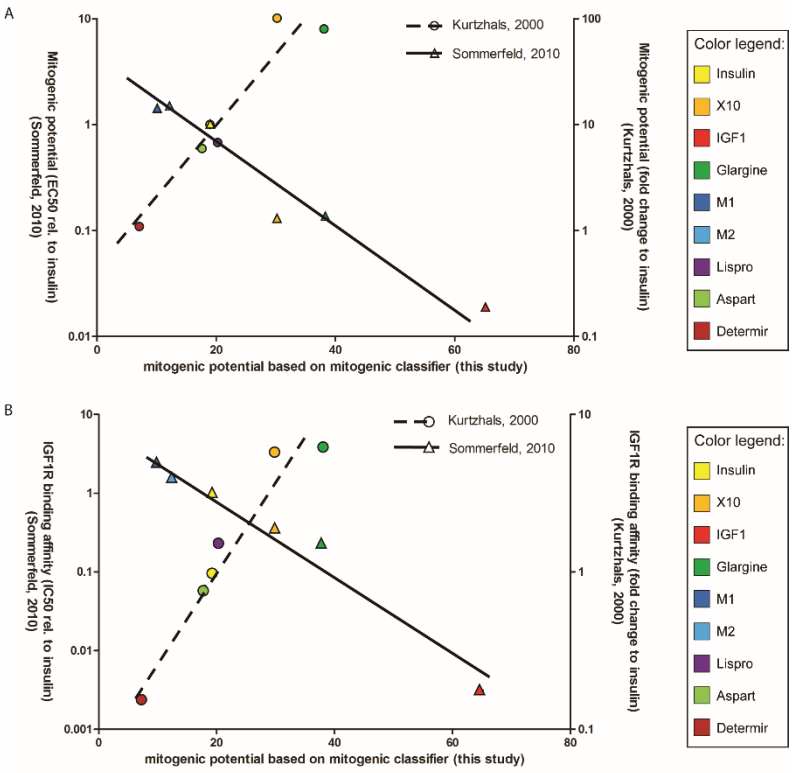


Figure 7. Correlation between mitogenic classifier score and the enhanced proliferation or IGF1R receptor binding. A) A correlation is presented of the mitogenic potential based on the mitogenic classifier analysis (x-axis) vs the mitogenic potential as determined by other studies with proliferation assays. Sommerfeld, 2010 expressed this score as EC50 value from their proliferation curves (left y-axis). Data from Kurtzhals, 2000 are expressed as fold changes compared to insulin (right y-axis). B) A similar correlation graph with the mitogenic potential from this study plotted against the IGF1R binding affinity according to two other studies. (data adapted from Kurtzhals 2000 and Sommerfeld 2010) [113, 114].

Previously we demonstrated that IGF1 and X10 significantly promote tumorigenesis in a conditional mammary gland tumor mouse model [153]. Glargine did not significantly enhance this tumorigenesis. Therefore we also evaluated the translation of our classifier genes to the *in vivo* situation and determined the gene expression changes in the mammary glands of mice that received a subcutaneous injection of the insulin-like molecules insulin, IGF1, X10 and glargine. We could validate the *in vitro* effect of IGF1 in the *in vivo* situation, indicating that a true IGF1R responses can be observed in this model. X10 showed some correlation with the effect of IGF1. Yet, in contrast to the *in vitro* data, the gene expression profiles of glargine were more similar to insulin than to X10/IGF1. This effect is very likely caused by the metabolism of insulin glargine by factors in the serum of the blood of the mice (similar to the glargine conversion in human serum). We therefore speculate that the observed pro-mitogenic signaling events of glargine in our *in vitro* breast cancer cell line models are presumably not occurring under *in vivo* conditions in the mammary gland. Yet, we cannot exclude that IGF1R mediated responses by glargine take place in other tissues in vivo.

Conclusions

In the current study we propose a new robust classifier gene set that allows the quick, robust and quantitative analysis of the pro-mitogenic potential of newly developed insulin analogues. These classifiers can be used within the pharmaceutical industry as well as in a regulatory setting to define the safety profile of insulin analogues as well as other growth factors that might act on the IGF1R.

Competing interests

There is no duality of interest that could be perceived as prejudicing the impartiality of the research reported. None of the authors declares any conflict of interest.

Authors contributions

Conceived and designed the experiments: BTB, CLES, SW, BVDW, JWVDL. Performed the experiments: BTB, EK, CP. Analysed the data: BTB, SW, EK. Wrote the paper: BTB. Reviewed and corrected paper: CLES, SW, EK, BVDW, JWVDL. All authors approved the final version of this paper.

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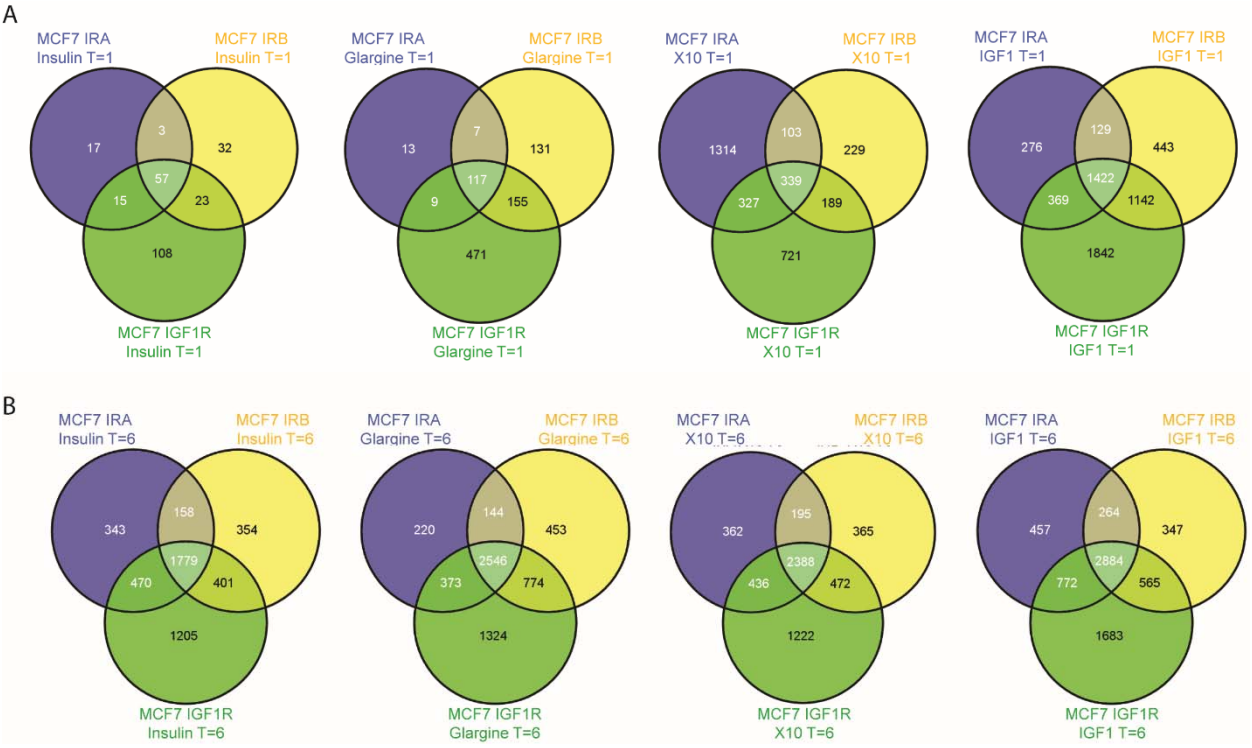
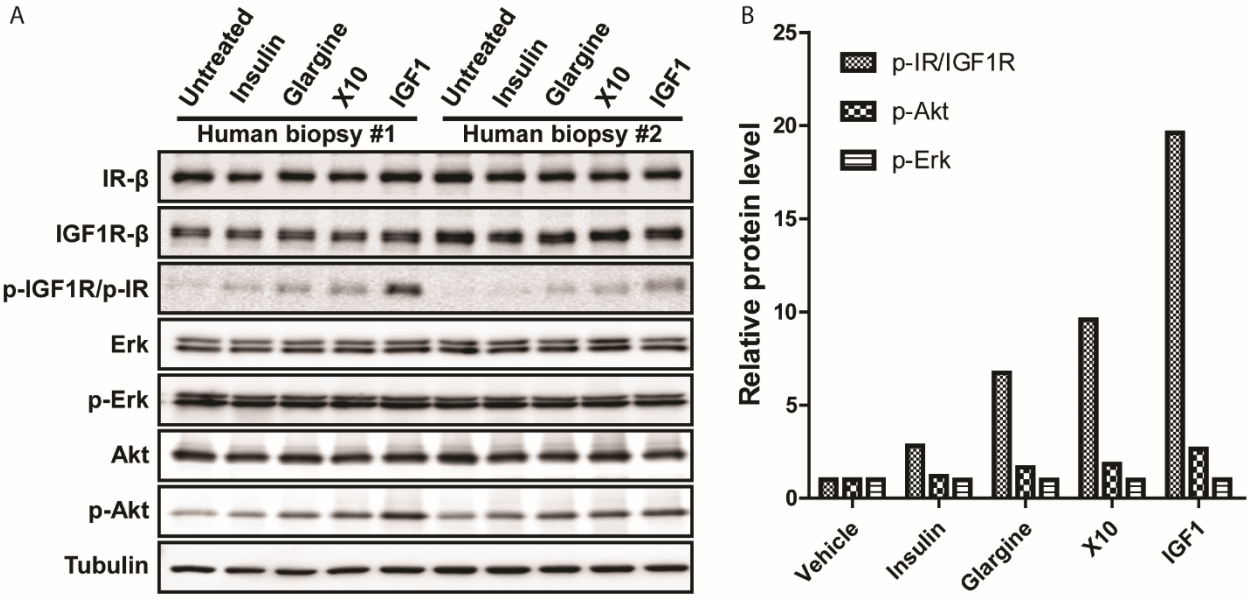
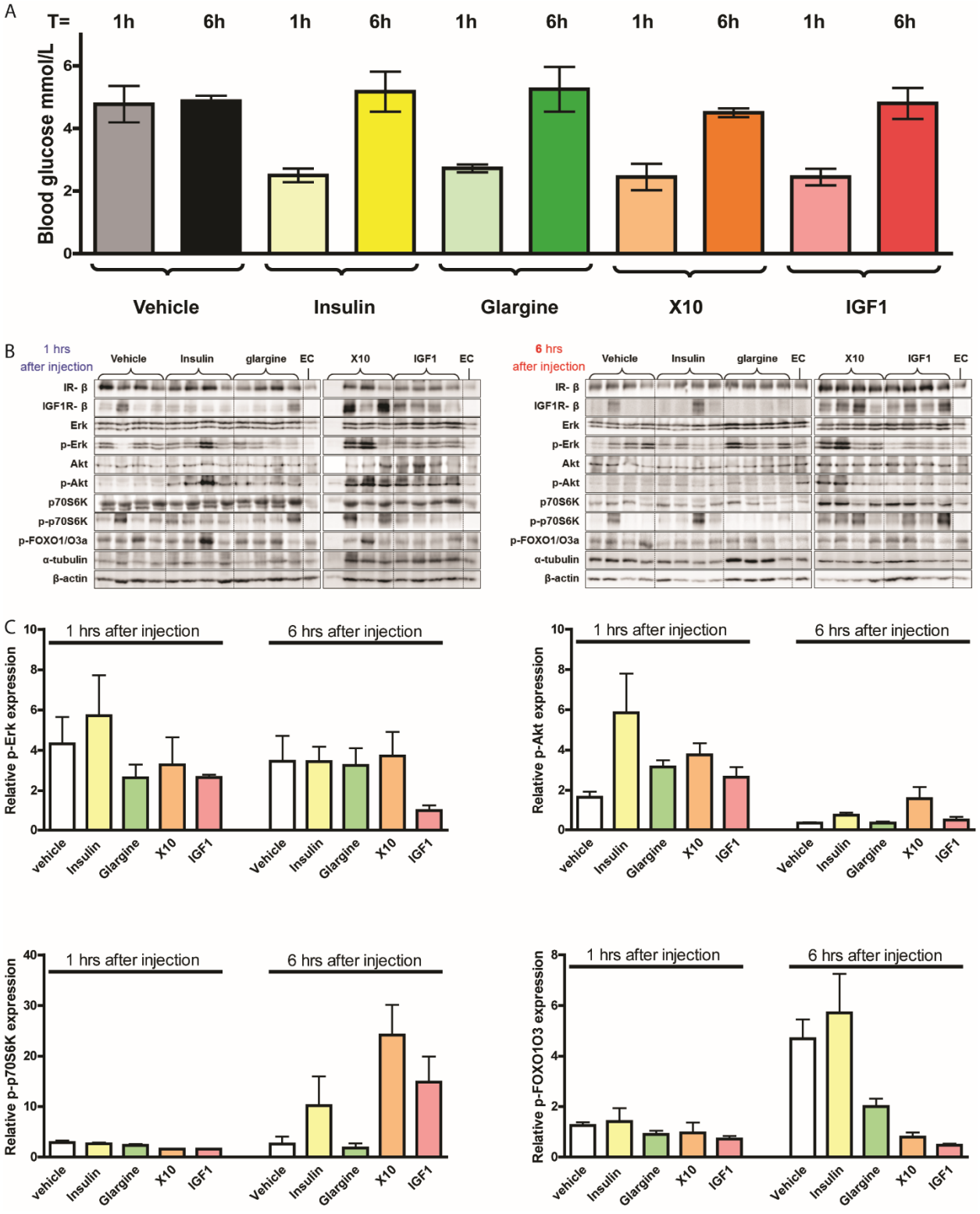
Supplementary figures



Supplemental Figure 1. Venn diagrams of the differentially expressed genes per cell line, time point and treatment showing overlap of genes between the different conditions. A) Venn diagram of the early regulators (t=1hr). the blue circle represents the hits in the IRA cell line, yellow the IRB cell line and green the IGF1R cell line. The first graph shows the number of hits after insulin treatment, the second graph glargine, the third X10 and the last graph shows the number of hits after IGF1 treatment. B) The Venn diagrams of the late regulators (t=6hrs).



Supplemental Figure 2. Protein levels of primary human mammary cells stimulated with insulin like compounds. A) Primary human mammary gland cells were treated with different insulin-like molecules followed by Western blotting for various INSR and IGF1R signaling pathway components. B) Quantification of Western blot data of the p-IGF1R/p-IR, p-Akt and p-Erk. The y-axis represents the average protein expression level compared to vehicle exposure.



Supplemental Figure 3. Mammary gland protein levels of 40 FVB mice subcutaneously injected with insulin-like compounds. A) The blood glucose levels (mmol/L) have been measured 1 hr and 6 hrs after subcutaneous injections with different insulin-like molecules. B) Western blotting of the receptors and kinases in the INSR and IGF1R pathway measured in the mammary gland tissue; samples were from 1 hour (left graph) or 6 hours (right graph) after the subcutaneous injections of the presented insulin like molecules. C) the quantification of activated kinases (Erk, Akt, p70S6K, FOXO1/O3) relative to the Endogous Control (EC), a sample that was loaded on every blot. (n=4)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Chapter 5

Mammary gland tumor promotion by chronic administration of IGF1 and the insulin analogue AspB10 in the p53$^{R270H/+}$WAPCre mouse model

Highlights

- Chronic AspB10 and IGF1 treatment significantly decrease the mammary gland tumor latency time in the p53$^{R270H/+}$WAPCre mouse model
- Mice chronically exposed to AspB10 and IGF1 develop mammary gland tumors with a sustained activation of mitogenic pathways
- Chronic glargine treatment did not significantly affect the tumor latency time, but mice injected with glargine developed more tumors with an enhanced mitogenic protein expression signature

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Mammary gland tumor promotion by chronic administration of IGF1 and the insulin analogue AspB10 in the p53$^{R270H/+}$WAPCre mouse model

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◀ IN THE PICTURE

Hypodermic needle and packaged insulin (Nobel Museum, Stockholm). The discovery of insulin in 1921 opened new possibilities in the treatment of diabetes. Frederick Banting and J.J.R. MacLeod received the Nobel Prize in physiology or medicine in 1923 for their discovery.

◀ IN BEELD

Injectie naald en een insuline verpakking (Nobel Museum, Stockholm). De ontdekking van insuline in 1921 opende nieuwe mogelijkheden voor de behandeling van diabetici. Frederick Banting en J.J.R. MacLeod ontvingen de Nobelprijs in fysiologie en geneeskunde in 1923 voor hun ontdekking.

Abstract

Insulin analogues are structurally modified molecules with altered pharmaco-kinetic and -dynamic properties compared to regular human insulin used by diabetic patients. While these compounds are tested for undesired mitogenic effects, an epidemiological discussion is ongoing regarding an association between insulin analogue therapy and increased cancer incidence, including breast cancer. Standard *in vivo* rodent carcinogenesis assays do not pick up this possible increased carcinogenic potential.Here we studied the role of insulin analogues in breast cancer development. For this we used the human relevant mammary gland specific p53$^{R270H/+}$WAPCre mouse model. Animals received life long repeated treatment with four different insulin (–like) molecules: normal insulin, insulin glargine, insulin X10 (AspB10) or insulin-like growth factor 1 (IGF1).Insulin-like molecules with strong mitogenic signalling, insulin X10 and IGF1, significantly decreased the time for tumor development. Yet, insulin glargine and normal insulin, did not significantly decrease the latency time for (mammary gland) tumor development. The majority of tumors had an epithelial to mesenchymal transition phenotype (EMT), irrespective of treatment condition. Enhanced extracellular signalling related kinase (Erk) or serine/threonine kinase (Akt) mitogenic signalling was in particular present in tumors from the insulin X10 and IGF1 treatment groups.These data indicate that insulin-like molecules with enhanced mitogenic signalling increase the risk of breast cancer development. Moreover, the use of a tissue specific cancer model, like the p53$^{R270H/+}$WAPCre mouse model, is relevant to assess the intrinsic pro-carcinogenic potential of mitogenic and non-mitogenic biologicals such as insulin analogues.

Keywords: IGF1, insulin glargine, carcinogenic potential, breast cancer, mouse model, p53$^{R270H/+}$WAPCre

Introduction

For two decades there is an intensive debate about the human risk for increased cancer incidence when using insulin analogues [108]. Insulin and insulin analogues act via the insulin receptor (IR), of which there are two isoforms, IRA and IRB, and to a lesser extent via the insulin-like growth factor 1 receptor (IGF1R). An increased residence time as well as an increased binding affinity of synthetic insulin-like molecules towards IRA and especially IGF1R might affect carcinogenesis [113, 149]. Downstream signalling via these insulin receptor family members occurs via distinct pathways. Upon stimulation with insulin analogues with high affinity for IRB the PI3K/Akt pathway is activated, which is related to the metabolic role of insulin [116]. In contrast, activation of the IGF1 pathway via IGF1R results in an upregulation of both the MAPK/Erk signalling cascade and an asymmetrical activation of the Akt pathway [135], which is directly related to the limited mitogenic effect of insulin. Stimulation of the IRA results in a similar downstream signalling

cascade to that observed after IGF1R activation [135]. Epidemiological studies indicate a strong association between expression levels of both IGF1 and its receptor (IGF1R) and cancer initiation/progression [170, 171]. This creates a situation whereby insulin analogues with increased affinity for IRA and/or IGF1R may increase the cancer hazard.

Current approaches to assess the intrinsic carcinogenic potential of insulin analogues are limited. The binding affinity of insulin analogues to both IR and IGF1R and their subsequent activation and overall mitogenic capacity are currently used as part of the risk assessment in terms of carcinogenic potential of newly developed insulin analogues. This *in vitro* assessment is limited by the variability in the cell lines, culture conditions and proliferation assays that are used [46] [172]. Furthermore, kinetic parameters like administration, distribution, metabolism and excretion cannot always be captured in these *in vitro* models. Some insulin analogues that are currently on the market have also been tested in two-year carcinogenicity studies in rats and/or mice [32] [173]. So far only insulin X10 (also called AspB10) has been indicated to increase the tumor incidence in the chronic rodent studies and consequently it never reached the market [32, 108, 173]. While most insulin analogues, including glargine [174, 175] [176], were negative in these chronic bioassays, several epidemiological studies showed an increased breast cancer risk [16, 76, 177] [17], which could not be observed by others [18, 19, 58, 67, 86, 143, 178, 179]. Altogether these data stirred concerns that growth factor-like insulin analogues are potential non-genotoxic carcinogens [24].

Genetically engineered mouse (GEM) cancer models constitute powerful, alternative methods to assess the carcinogenetic potential of non-genotoxic compounds [180] . This in particular involves GEMs with constitutive or conditional tissue specific deletion of tumor suppressor genes [181]. We described a mammary gland specific dominant negative mutated p53 mouse model, $p53^{R270H/+}$WAPCre [182]. The model is based on a point mutation corresponding to the p53 mutated hotspot p53.R273H in the human *Li Fraumeni* cancer syndrome. Mutant p53 is only expressed when Cre recombinase is induced by the whey acidic protein promoter (WAP) in the mammary gland. This leads to spontaneous mammary gland tumor formation initiated within a year.

Here we used $p53^{R270H/+}$WAPCre model to evaluate the carcinogenic potential of several insulin (like) molecules: insulin NPH, insulin glargine, insulin X10 and IGF1. We demonstrated that chronic exposure to insulin X10 and IGF1 significantly promote mammary gland tumor development, while glargine and insulin do not. Yet, glargine-related tumors do have a different pro-mitogenic signalling that is distinct from control and insulin treated mice, and more reminiscent of insulin X10 and IGF1.

Methods

The p53$^{R270H/+}$WAPCre mouse model

Heterozygous p53.R270H as well as the WAPCre mice were backcrossed with FVB mice over 15 times to yield a > 99.99% FVB genetic background. >8 Week old heterozygous conditional p53.R270H mice were crossed to transgenic WAPCre mice of the same age to generate p53$^{R270H/+}$WAPCre mice [182] [183]. The mammary gland specific Cre recombinase splices out the intronic floxed stop cassette of the p53.R270H allele that eventually would lead to spontaneous mammary gland tumor formation of 1 year old p53$^{R270H/+}$WAPCre female mice. A high expression the WAP promoter was used [184], which is already active in the mammary gland of non-pregnant, non-lactating virgin mice. Therefore we used only nulliparous mice in this experiment. PCR/digestion based assay was used for genotyping using the same primers as described [182] and subsequent restriction analysis using Hsp92II. Presence of the R270H mutation leads to digestion of the 486 bp p53 PCR amplicon into a 269 and 217 bp product. Presence of Cre recombinase was verified by a 676 bp amplicon [182]. All mice were fed *ad libitum* with RM1 diet (SDS, technilab-BMI, Holland).

Preparation of insulin, insulin analogues and IGF1 injection solutions

The treatments included: insulin NPH (Insuman Basal, Sanofi Aventis), insulin glargine (Lantus, Sanofi Aventis), insulin X10 (AspB10, Novo Nordisk) and IGF1 (Increlex, Ipsen). All compounds were dissolved in their original vehicle solutions: glargine (glycerol 0.2 mol/l, m-cresol 0.025 mol/l, ZnCl2 0.0002 mol/l adjusted to pH 4.0), insulin (glycerol 0.2 mol/l, NaH2PO4 0.00135 mol/l, phenol 0.0063 mol/l, m-cresol 0.0138mol/l,ZnCl2 0.0001mol/l adjusted to pH 7.4), X10 (glycerol 0.2 mol/l, phenol 0.0063 mol/l, m-cresol 0.0138mol/l, ZnCl2 0.0001mol/l adjusted to pH 7.4) and IGF1 (benzyl alcohol 0.083 mol/l, sodium chloride 0.1 mol/l, polysorbate 20 0.0016 mol/l, acetic acid 0.0072 mol/l, sodium acetate 0.05mol/l adjusted to ph 5.4).

Experimental set-up

The experimental setup of the studies was examined and approved by the institute's Ethical Committee on Animal Experimentation, in accordance with national and European legislation.

In a first panel of short term experiments, the maximal pharmalogical dose (MPD) was determined for each compound in our mouse model. In a 10 hr experiment (n=54) the glucose drop was measured (Freestyle light, 70812-70, Abbott) sequentially every hour after a single subcutaneous injection with insulin NPH, glargine, regular insulin, X10 or IGF1 (Supplemental Figure 1). A wide concentration range based on literature was evaluated: insulin NPH and insulin glargine (25 nmol/kg – 125 nmol/kg); insulin X10 (480 nmol/kg – 1800 nmol/kg); and IGF1 (5 mg/kg – 15 mg/kg) [174]. In a subsequent experiment (n=52) the effects of frequent injections were determined (Supplemental Figure 2). Besides blood glucose levels, the weight and overall well-being were determined during one month of daily injections.

Based on the above data the long-term exposure experiment was designed in which the mice were injected with 50% and 80% of the MPD according to Supplemental Table 1. Once the female $p53^{R270H/+}$WAPCre mice were about 8 weeks old, they were randomly distributed in the dose groups using the program "randomice". Mice were weighed every week, and a standard injection solution per compound/concentration was made to ensure an injection volume in the range of 60-130 uL. To avoid adverse reactions at the injection site due to frequent injection, three subcutaneous injection sites were used: neck, upper back and lower back. Mice were injected every other day for up to 67 weeks and palpated for tumors twice a week. A typical mammary gland tumor could be detected once it had a volume of about 8 mm^3. Dimensions were noted to monitor tumor growth. Once the tumor reached a volume of about 1 cm^3, the mouse was sacrificed and dissected. Tumors from other origins were generally difficult to palpate, so other fitness markers (weight loss, skin condition, motility etc.) were used to decide when to sacrifice an animal.

Histopathology and immunofluoresence

When mice were destined for sacrifice based on tumor size or other markers, one day after the last injection mice were euthanized, blood was collected and serum was extracted (mini collect, Greiner/Omnilabo) according to manufactures protocol. ¼ of tumor, liver, lung, pancreas, kidney and spleen were fixed in a neutral aqueous phosphate buffered 4% solution of formaldehyde (Klinipath/VWR) for 24 hrs and stored in 70% EtOH. These tissues were embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E) for histopathologic evaluation. Immunofluorescence (IF) was performed on twenty representive tumors (based on the H&E characterisation) from the different tumor types. Citrate buffer was used for antigen retrieval, blocking was performed in 10% normal goat serum (NGS). Antibodies were diluted (1:10 – 1:800) in 1% NGS buffer. Antibodies used were smooth muscle actin (A2547, Sigma), cytokeratin 5 (PRB-160P, Covance), cytokeratin 8 (rdi-pro61038, Fitzgerald/Bioconnect), E-cadherin (610181, BD Transduction). Fluorescently labelled secondary antibodies were from Jackson Laboratories. Images were made on a Nikon Eclipse TS100 microscope. The histopathology was based on analysis of the macroscopic tumors. The epithelial and mesenchymal cell distributions were estimated by a pathologist on morphological characteristics of these cell types.

Western blotting

A quarter of the tumor was snap frozen in liquid nitrogen, stored at -80˚C and used for subsequent protein expression profiling. A tumor piece was grinded and lysed with 300 µL tumor lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton, 10% glycerol, 1:100 protease inhibitor cocktail) for 30 minutes at 4°C. After centrifugation (10 min, 10,000 rpm) supernatant was collected and the protein concentration was determined (BCA TM Protein Assay

Kit; Thermo Scientific, Rockford, IL, USA). The Western blotting procedures and all antibodies were identical to [46]. Furthermore, antibodies targeting Her2 (Cell Signalling Technology, Danvers, MA, USA), β-Actin, GAPDH, ER-α and EGFR, (Santa Cruz Biotechnology, Santa Cruz, CA, USA) have been used. The same endogenous control (EC) sample consisting of a mix of 30 random mammary gland tumor samples was used on each blot.

Statistical analysis

Statistical analyses of (mammary gland) tumor-free survival curves included calculation of censored Kaplan-Meijer distribution of survival of two different treatment groups and comparison by a two-sided log-rank test using Graphpad Prism version 4.00 software. The same software was used to determine p-values over de mean weight, % of epithelial cells and average number of mammary gland tumors using the two-sided t-test. For the correlation plots linear regression was applied.

Results

Insulin analogue treatment induces a metabolic and at high concentrations a toxic response

We first systematically determined the doses at which each insulin analogue induced a glucose drop (Supplemental Figure 1). Two hours after the injection the maximal (approximately 60%) blood glucose decrease was observed (from ~6 to ~2 mmol/L) which was followed by a glucose recovery, depending on the insulin dose. Mice injected with short-acting insulin forms (X10 and regular insulin) required more time to recover. Next, a dose dependent positive weight effect was observed during one month daily treatment for most compounds; only the highest dose of insulin NPH and IGF1 caused a weight loss compared to control (Supplemental Figure 2). Despite this evident adverse effect, no mortality of mice was observed so this dose was referred to as the MPD.

Insulin X10 and IGF1 treatment of $p53^{R270H/+}$WAPCre female mice increases body weight

We then performed a life long repeated dosing of 10-week old $p53^{R270H/+}$WAPCre female mice with all four insulin analogues as well as the controls. We carefully monitored the weight up till 35 weeks, a point in time at which the first animals developed mammary tumors (Figure 1A, top panel). X10 and IGF1 induced the strongest weight gain. In contrast, insulin and glargine did not affect the weight compared to the vehicle treated animals.

Insulin X10 and IGF1 treatment significantly decreases the latency time for mammary gland tumor development

Next we further followed mammary gland tumor formation. In total 100% of the mice that remained part of the experiment developed tumors, 83% of which were mammary gland tumors with an average latency time of 40 weeks (Figure 1B and Table 1).

Subcutaneous injections of vehicle 1 and vehicle 2 did not significantly cause a difference in tumor latency time compared to untreated animals (p=0.7194, data not shown) and were further combined in one control "vehicle" group. Subcutaneous injection of the vehicle did not significantly affect the tumor latency time compared to untreated animals (p=0.8476, data not shown). Each insulin analogue was dosed at 50% and 80% of the MPD. None of the different compounds showed a difference between the two doses (insulin p=0.1839, glargine p=0.1447, X10 p=0.1619, IGF1 p=0.6064; data not shown). Therefore we also combined the two dose groups per compound. The tumor growth rate was next determined by calculating the tumor doubling time in the exponential part of the Gompertz equation curve as in [185]. All tumors showed a similar doubling time (1.44 -1.78 day^{-1}), independent of treatment conditions (Table 1).

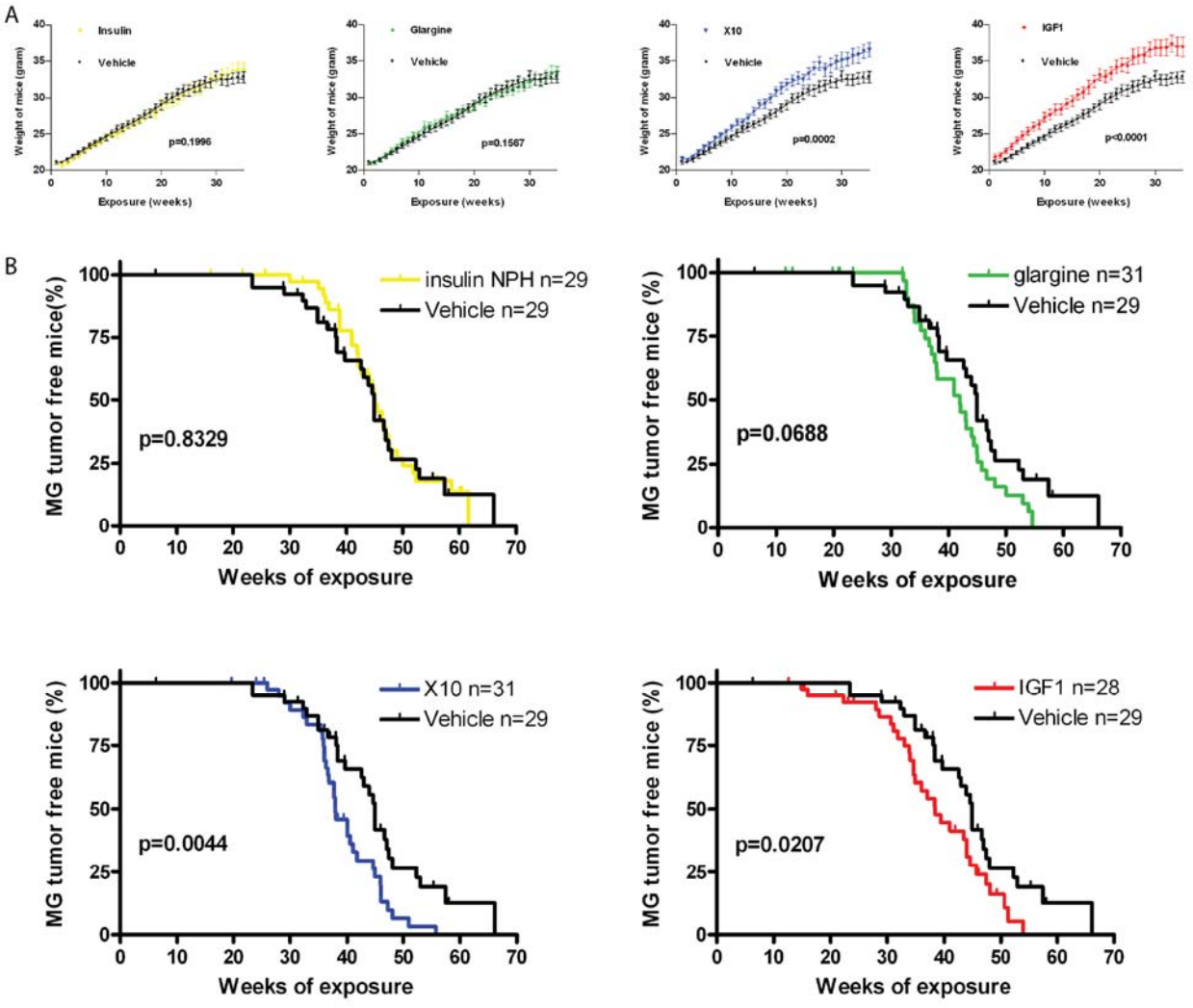

Figure 1. Effect of insulin analogues on mammary gland tumor development in p53$^{R270H/+}$WAPCre female mice. A) Upper graphs represent the average weight of the mice in the indicated groups during the first 35 weeks of the experiment. Statistical analysis was determined with an unpaired T-test. B) Effect of different compounds on mammary gland tumor free survival shown as in Kaplan Meier curves. Statistical analysis for the survival curves was determined with logrank test.

Table 1. Results overview of p53$^{R270H/+}$WAPCre model subcutaneously injected with growth factors

	Insulin	Glargine	X10	IGF1	Vehicle
Number of mice analyzed	40	40	40	40	38
Mortality	6/40 (15%)	3/40 (8%)	5/40 (13%)	4/40 (10%)	1/38 (3%)
Mean weight of mice (grams) *	30 ± 0.5	30 ± 0.5	32 ± 0.6	34 ± 0.7	29 ± 0.4
p-value#	0.1996	0.1567	0.0002	<0.0001	NA
Tumor-bearing mice	34/34 (100%)	37/37 (100%)	35/35 (100%)	36/36 (100%)	37/37 (100%)
Mean tumor latency-time (wks) †	44.9	38.0	37.9	36.0	42.6
p-value‡	0.1463	0.1448	0.0707	0.0549	NA
Mammary gland tumor	29/34 (85%)	31/37 (84%)	31/35 (89%)	28/36 (78%)	29/37 (78%)
Carcinocarcoma	24/29 (83%)	25/31 (81%)	26/31 (84%)	25/28 (89%)	23/29 (79%)
EMT status (% epithelial)	20.8 ± 4.89	27.5 ± 4.83	31.2 ± 4.01	18.8 ± 4.71	15.71 ± 3.80
p-value#	0.2170	0.0672	0.0086	0.6251	NA
carcinoma	5/29 (17%)	5/31 (16%)	5/31 (16%)	3/28 (11%)	6/29 (21%)
Adenoma	0/29	1/31 (3%)	0/31	0/28	0/29
Mean MG tumor latency-time (wks) †	45.0	41.9	37.9	38.4	44.9
p-value‡	0.8329	0.0688	0.0044	0.0207	NA
Mean MG tumor doubling time (days)	1.75 ± 0.11	1.87 ± 0.21	1.83 ± 0.19	1.53 ± 0.12	1.470 ± 0.14
p-value#	0.1375	0.1305	0.1632	0.7594	NA
Mice with multiple MG tumors	10/34 (29%)	16/37 (43%)	18/35 (51%)	16/36 (44%)	13/37 (35%)
Average number of MG tumors	1.50 ± 0.16	2.00 ± 0.24	1.82 ± 0.16	2.07 ± 0.23	1.64 ± 0.15
p-value#	0.5289	0.2371	0.4337	0.1346	NA

***** Mean weight depicted as average weight of mice from start of injections (mice are ± 8 wks) till mice are sacrificed.

† Mean latency time is depicted as number of weeks after start of the subcutaneous injections.

‡ p-values determined by comparing the Kaplan-Meijer curves of compounds with vehicle

p-values determined by comparing compounds with vehicle. Unpaired T-test.

The overall mammary gland tumor latency time (MTLT) for control mice was 44.9 weeks. IGF1 and insulin X10 caused a significantly decreased mean MTLT of 6,5 and 7,0 weeks respectively, compared to the vehicle treatment (Figure 1B; MTLT decreases from 44.9 weeks for control to 38.4 weeks (p=0.0207) and 37.9 weeks (p=0.0044) for IGF1 and X10, respectively). Glargine injections led to a tumor latency time reduction of three weeks, although this was not significantly different (p=0.0688). No effect on MTLT was observed for treatment with human insulin (p=0.8329). 41% of the mice developed multiple mammary gland tumors.

Different insulin analogues do not affect mammary gland tumor type

All mammary proliferative lesions were next classified according to the criteria described by the Annapolis Pathology Panel (Figure 2A) [186]. Irrespective of treatment condition, the majority (123/148, 83%) of mammary gland tumors was diagnosed as EMT (Epithelial to Mesenchymal Transition) tumor, 16% (24/148) as carcinoma, and 1% (1/148) as adenoma (Table 1).

EMT tumors with a clearly distinguishable histological epithelial component also showed clear E-cadherin staining, whereas EMT tumors that predominantly consist of mesenchymal cells showed high levels of SMA (Figure 2B). In total 31 mice developed other tumors: 10 thymic lymphoma, 6 lung adenocarcinomas, and a small number of lymphomas, bone, uterus or pancreas tumors (Figure 2C). No correlation between the prevalence of non-mammary gland tumors and treatment could be detected. Typically lymphomas developed significantly earlier (independent of treatment) compared to mammary gland EMT tumors and carcinomas (30.8 wks vs 41.4 and 39.1 weeks respectively).

There was no clear correlation between treatment and tumor spectrum (Figure 2D). EMT tumors are previously described as carcinosarcomas, and these terms can be used as synonyms[187]. 3D culturing of isolated cells from these primary tumors resulted in an invasive morphology, one of the characteristics of an EMT tumor (data not shown). The EMT-phenotype tumor was considered morphologically significant in the presence of epithelial structures displaying loss and disruption of basement membrane and associated with epithelial-to-spindle cell morphology transition and gradual blending or invasion into the surrounding stroma [188]. Most mammary tumors showed invasion of the surrounding fat pad. Variable-sized intratumoral areas of necrosis were also present, often accompanied by desmoplastic reaction and severe infiltration of inflammatory cells composed by mostly neutrophils and reactive macrophages.

Some mice developed more than one mammary gland tumor. There appears to be a trend in the incidence of increased multiple mammary gland (MG) tumors for glargine, X10 and IGF1, compared to vehicle treatment, but none of these treatments reached significance (Figure 2E).

We also assessed the percentages of epithelial and mesenchymal cells. Only X10 treatment induced significantly ($p=0.0086$) more EMT tumors with a higher epithelial prevalence (Figure 2F). To further assess the origin from either the basal or the luminal part of the mammary gland ducts, the expression of CK5 and CK8 was evaluated, respectively. Based on expert judgement of the pathologist we have screened a subset of twenty tumors, equally distributed over the treatment groups. From these tumors only one was stained positive for CK5 and negative for CK8, all other tumors showed clear CK8 staining and lack of CK5 indicating that the majority of tumors likely originated from the luminal part of the mammary gland. A representative immunofluorescent picture of a luminal tumor and the only basal tumor that we found can be

found in Supplemental Figure 3. Neither a correlation was detected between the different treatment conditions, nor with tumor histology.

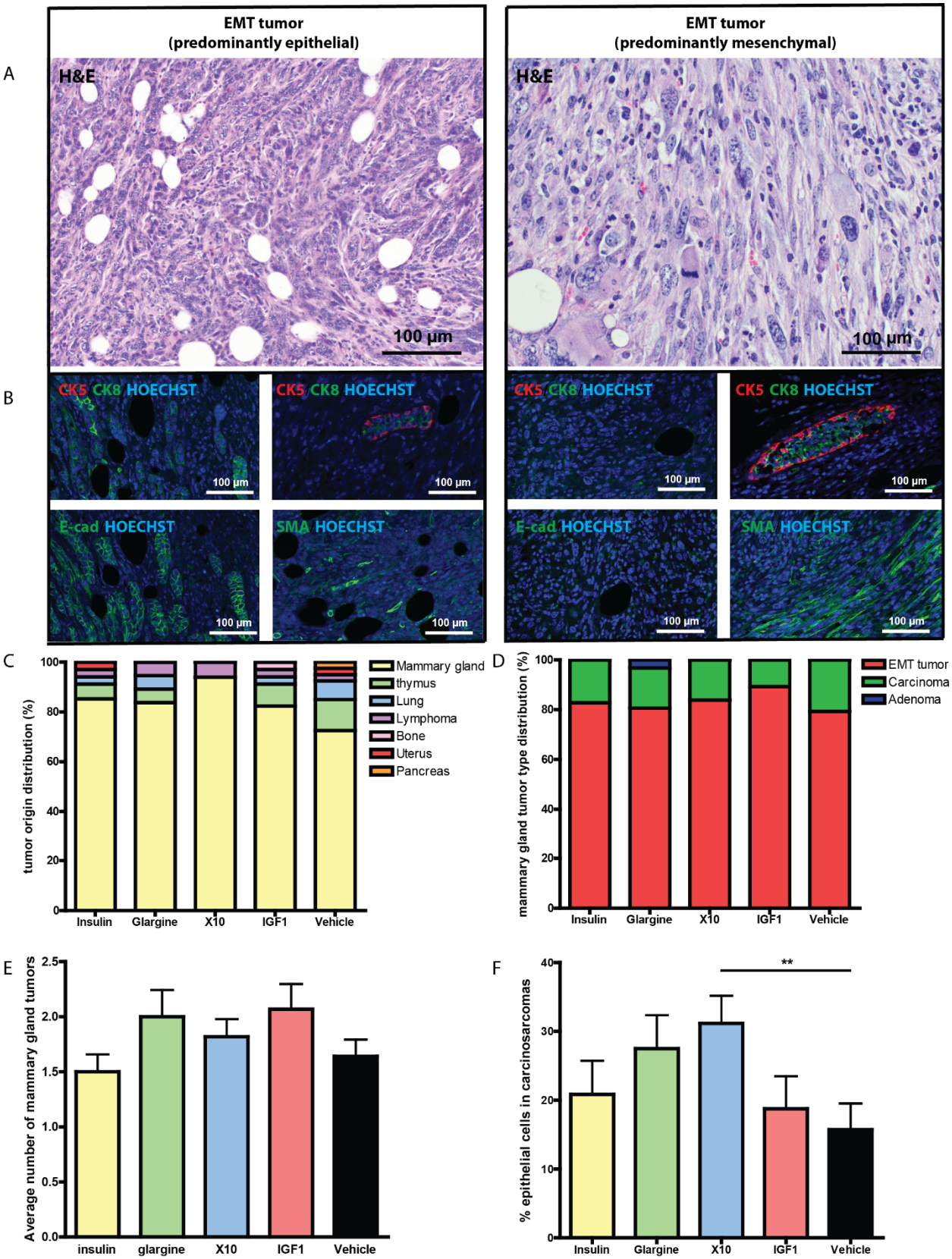

Figure 2. Insulin analogues induce primarily EMT tumors in p53$^{R270H/+}$WAPCre female mice. A) H&E images of representative EMT tumors with predominantly epithelial cells (left panel) and an EMT tumor with predominantly mesenchymal cells (right panel). B) Immunofluoresent images of CK5/8, area with normal mammary duct on the same slide that serves as control for CK5/CK8 staining, E-cadherin and smooth muscle actin. C) Tumor type distribution per treatment. D) Mammary gland tumor type distribution per treatment. E) Average number of mammary gland tumors. F) Percentage of epithelial cells in the EMT tumors over the different treatment groups.

Molecular profiling of IR and IGF1R signaling pathways in insulin analogue induced mammary gland tumors

To further understand the underlying mechanism of the decreased tumor latency by IGF1 and X10, we determined the expression of proteins in the main signalling pathways associated with IR and IGF1R signalling as well proteins used in breast cancer classification. This included the quantitative analysis of IR-β, IGF1R-β, EGFR, Her2, E-cadherin, N-cadherin, Akt, p-Akt, Erk, p-Erk, ER-α, tubulin and β-actin protein levels in all primary mammary gland tumors. A small subset of the Western Blot data (n=36) is presented in Figure 3A, the quantitative analysis of Figure 3B is based on all Western Blots (n=148) shown in Supplemental Figure 4. Overall only IR, IGF1R, p-Erk or p-Akt levels were significantly affected in insulin analogue treatment-related mammary tumor (Figure 3B). Interestingly, the IR and IGF1R receptor levels in tumors of compound treated animals were all significantly upregulated compared to control. Importantly, IGF1-dependent tumors did not only upregulate IGF1R, but also showed a significant (p=0.0066) upregulation of IR; similarly insulin treated mice demonstrated IR upregulation but also showed significantly (p=0.0005) higher IGF1R levels. Also phosphorylated Akt was more abundant in tumors of mice treated with insulin-like molecules, especially insulin, X10 and IGF1 stimulation led to tumors with very significant higher activation of the PI3K signalling pathway. Interestingly, glargine, X10 and IGF1 but not insulin stimulation led to an increased activation of the MAPK signalling cascade in the obtained tumors. This effect was only significant for glargine ($p = 0.0125$) and X10 ($p = 0.0018$) but not for IGF1 ($p = 0.0980$) when compared to the tumors from the vehicle treated mice. We also compared the association between IR and IGF1R expression (Figure 3C). Overall there seemed a direct linear correlation between IR and IGF1R, suggesting interdependency. To evaluate the relationship between IR and IGF1R levels and tumor latency, we separated the entire group of tumors in five equal clusters each representing 20% of the tumors based on right-angled distribution on the linear regression curve. For each cluster the mean latency time was determined irrespective of treatment condition (Figure 3D), but only involving insulin, glargine, X10 and IGF1 conditions. Intriguingly, a long latency time, and thus a long exposure to the insulin-like molecules, is associated with increased insulin- and IGF1- receptor levels. While the tumors of all control animals showed a strong correlation between IR and IGF1R expression levels, albeit at lower expression level (see Supplemental Figure 4 and Figure 3B), no correlation was observed

with either latency time or receptor levels. A similar correlation was observed between IR/p-Akt and IGF1R/p-Akt (data not shown).

Mitogenic insulin analogue tumor formation is related to enhanced Erk and Akt activity.

Since the insulin analogue treatment apparently affected and directed the composition and the activity of signalling machinery in the mammary gland tumors, we next determined whether specific subgroups of profiles existed and whether these would cluster with particular insulin analogues. We focused on those signalling components that were significantly different between control and insulin-like molecule treatment (i.e. IR, IGF1R, p-Erk and p-Akt) and evaluated all EMT tumors (n=123; the major tumor type irrespective of treatment condition), thereby eliminating tumor type variation. Hierarchial clustering of the quantitative signalling data separated in total nine clusters (Supplemental Figure 4A and 4B). Five clusters showed increased p-Erk level as a dominating signalling pathway: clusters 1, 5 and 7 represented high p-Erk which was dominated by IGF1, X10 and glargine related EMT tumors (together 78 %, n=22 tumors); clusters 2 and 8 showed lower p-Erk with no clear enrichment (n=15 tumors). In addition, especially two clusters were representing Akt activation: clusters 5 and 6 preferentially showed high p-Akt activity, and together these clusters demonstrated enrichment for IGF1 and X10 treatment (75 %; n=16 tumors). Importantly, there were no tumors from the control group that showed high p-Akt levels. Two large clusters (4 and 9) did not show any dominance or activation of either Erk or Akt; in these two clusters there was a slight overrepresentation of control and insulin treatment (61 %; n=31 tumors). These combined data suggest that pro-mitogenic insulin analogues drive tumor formation that are dominated by either p-Erk and/or p-Akt, although at this point we cannot exclude the activation of additional signalling pathways. We also determined for each cluster the tumor latency time (Figure 4C). Overall, clusters enriched for pro-mitogenic insulin analogues and associated enhanced mitogenic signalling, either Erk or Akt, did not significantly correlate with latency time. Yet, a tendency existed for a shorter latency time in cluster 1 which had the highest p-Erk level. Therefore, we also determined the correlation between either p-Erk or p-Akt status for any treatment condition and mammary gland tumor latency time (Figure 4D and 4E). A significant negative correlation was found between p-Erk status and latency time. This effect was strongest for IGF1 induced tumors, but glargine induced tumors also showed this trend. A weak non-significant positive correlation was found between p-Akt status and EMT tumor latency time. Interestingly, this positive effect was observed for all treatments, except for the control group.

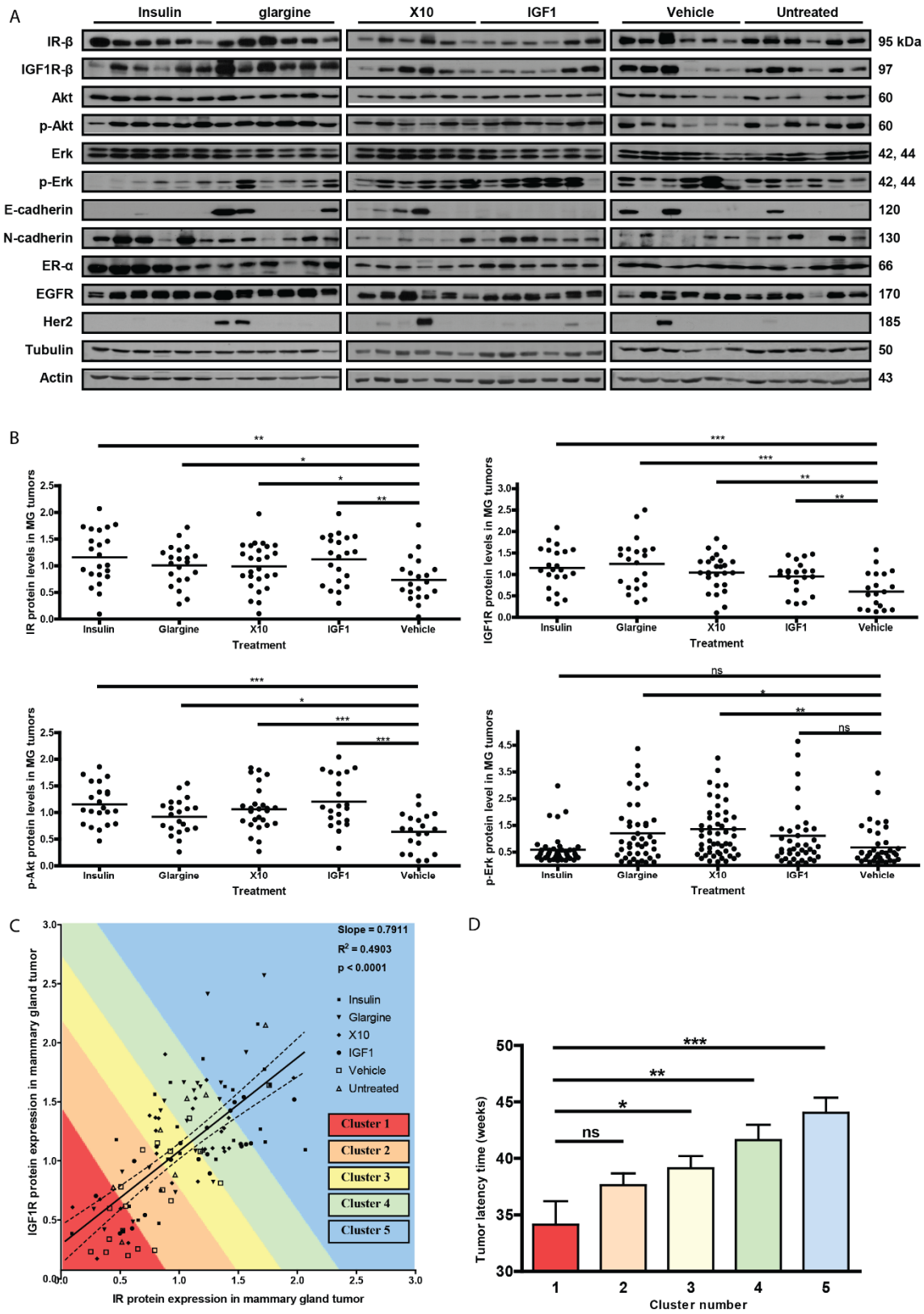

Figure 3. Molecular profiling of IR and IGF1R signaling in insulin analogue derived mammary gland tumors. Tumor protein levels of critical mammary gland tumor related receptors (IR, IGF1R, ER, EGFR, Her2) and downstream signaling pathways (Erk, phospho-Erk, Akt, phospho-Akt) as well as epithelial differentiation markers (N-cadherin and E-cadherin) were determined by quantitative Western blotting of all primary mammary gland tumors (n=148). A) A small subset of the Western Blot data (n=36) is shown which are representative for the quality of the blots. B) The quantitative IR, IGF1R p-Akt and p-Erk levels of all primary EMT tumors are presented in dot-plots (n=148). C)

The IR vs IGF1R protein levels are plotted, 5 clusters are defined. D) The mean tumor latency time has been determined of each cluster. Error bars represent SEM, ns = p>0.05, *= p<0.05, **= p<0.01, ***=p<0.001.

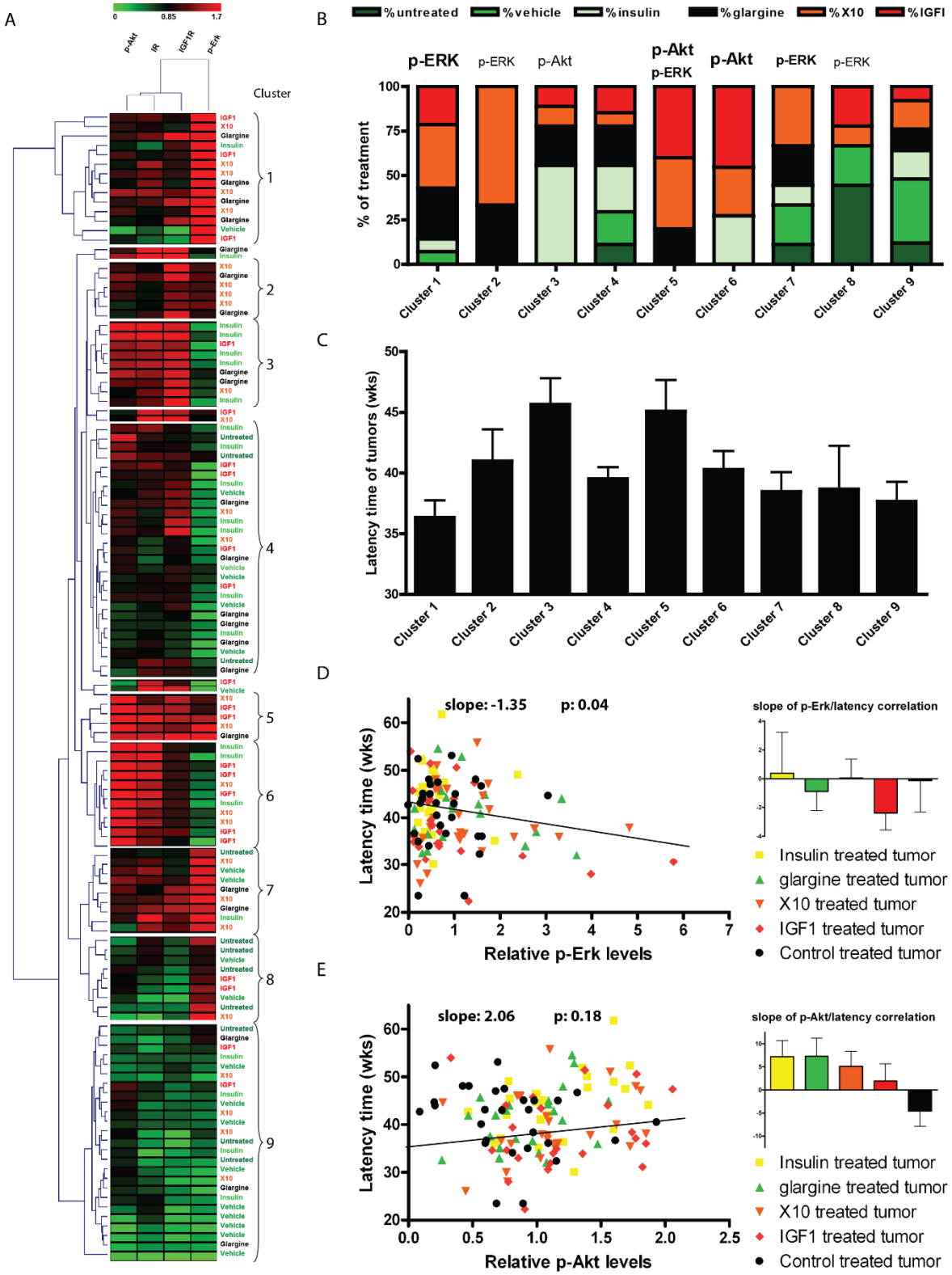

Figure 4. Hierarchical clustering of insulin (analogue) tumors based on IR and IGF1R signalling components. Quantitative expression levels of IGF1R-β, IR-β, p-Akt and p-Erk in mammary gland EMT tumors of all treatment groups were clustered using Euclidean distance and average linkage (A). Distinct clusters appeared in which the treatment groups are not equally distributed. In protein clusters 1, 2, 5 and 6, the highly mitogenic treatment groups

(IGF1 and X10) are overrepresented. In the graphs B and C the distribution of several parameters of the clusters are shown. B) Treatment group distribution, C) Latency time per cluster, in which it became apparent that the cluster with the highest p-Erk levels has the shortest tumor latency time. In the last two graphs the correlation between latency time and p-Erk (D) or latency time and p-Akt (E) is presented. Interestingly, high p-Akt levels are positively correlated with latency time, but only in the compound treatment groups and not in the vehicle treated animals.

Discussion

In the present study we used the $p53^{R270H/+}$WAPCre mouse model to assess the carcinogenic potential of two insulin-like molecules, X10 (AspB10) and glargine in direct comparison to insulin NPH and IGF1. Our data indicate that the highly mitogenic compounds insulin X10 and IGF1, both stimulating the IGF1-receptor, significantly decreased the latency time for tumor development. This was not observed for glargine and insulin NPH. Moreover, we demonstrated that tumors derived after mitogenic insulin analogue treatment induce mammary gland tumors with enhanced intracellular signalling through either the Erk or Akt pathway, which was not observed in control animals. Our data indicate that the $p53^{R270H/+}$WAPCre mouse model is sensitive to evaluate the intrinsic higher mitogenic potential of insulin-like compounds and the associated contribution to cancer development. Interestingly, while this mouse tumor model gives largely rise to EMT mammary gland tumors irrespective of insulin treatment conditions, the mitogenic insulin-like molecules drive the formation of tumors with enhanced key mitogenic signalling activity.

X10 as well as IGF1 significantly decreased tumor latency time. These two compounds also induced a significant weight increase. However, there is no overall correlation between tumor latency time and mouse weight at tumor detection date (Supplemental Figure 5), indicating that chronic treatment of X10 and IGF1 affects tumor latency directly by mitogenic signalling rather than indirectly by obesity.

Similar to insulin NPH, chronic exposure to insulin glargine did not significantly affect tumor latency time compared to the vehicle treatment, although tumors developed slightly earlier in the glargine treated animals. These glargine data are in line with a previous report by Stammberger et al. in which a life long exposure of glargine did not show any difference in the incidence of mammary tumors reported in both mice and rats when comparing with the NaCl, vehicle-control, or the NPH insulin treated groups in wild type mice and rats [175]. When conducting a pathology study on the different tumors, we found no difference between insulin NPH versus glargine induced tumors. These histopathology results are in agreement with a study performed by Besic et al, in which a clinical and histopathological screening was performed on breast carcinomas of diabetic patients that were either on a glargine or other insulin (analogue) therapy [93]. We previously showed in *in vitro* models that both insulin IGF1, X10 and glargine can strongly activate both the IGF1R-mediated signalling, but that only IGF1 and X10 have an increased mitogenic potential [46]. Insulin glargine did not significantly enhance cell proliferation, which was largely explained by the rapid

metabolism of glargine, preventing a sustained activation of IGF1R-mediated mitogenic signalling [46]. These insulin-like compounds have been tested for carcinogenic side-effects in wild type mice and rats [32, 189] [173], but to our knowledge they have never been tested in sensitive humanized *in vivo* models. Our current *in vivo* findings are in agreement with these *in vitro* observations. Yet, despite the fact that insulin glargine did not significantly enhance mammary gland tumour development, many insulin glargine tumours also demonstrated enhanced Erk and Akt activity, which was hardly observed under control conditions. This suggests that insulin glargine is not inert and may affect the intracellular signalling in the developing tumor.

The majority of the obtained tumors in our $p53^{R270H/+}$WAPCre mouse model were classified as EMT tumors, these tumors are thought to be in the epithelial to mesenchymal transition state. This type of mammary gland tumor is not a commonly found human breast cancer subtype. Nevertheless, like in human breast cancer, the intracellular signalling varied considerably between the various tumors. In particular a strong enhancement of Erk and Akt activity was evident in the mitogenic insulin like molecules groups, which was hardly observed for untreated mice. This variation induced by our insulin treatment conditions probably provides a better representation of human breast cancer in general and EMT tumors in particular. Hence, a mitogenic treatment setting might provide enhanced information on human breast cancer development. Yet, given the biased formation of EMT tumors in the current study setup, further carcinogenic studies with insulin-like molecules are required with other humanized mouse mammary gland tumor models. This could involve genetically engineered mouse models (GEMMs) with a human specific mutation in *e.g.* the PI3K signalling pathway [190].

Long-term administration of IGF1, X10, glargine and insulin, led to tumors with significantly higher p-Akt levels compared to vehicle treated animals. This indicates that the PI3K signalling cascade is up regulated upon stimulation with the insulin-like molecules. Similarly the MAPK signalling pathway was up regulated after IGF1, X10 and glargine treatment. Interestingly long-term stimulation with insulin did not affect the p-Erk1/2 levels in the obtained tumors.

At this moment we do not know the exact mechanism by which the more mitogenic insulin like molecules promote the mammary gland tumor development. Since Erk and Akt activity did not per se coincide in the various tumors and/or relate to enhanced IGF1R levels, direct ligand-mediated activation of the Erk and Akt pathways seems unlikely. Possibly mutations in either Erk and/or Akt pathway components are underlying the enhanced activation of these signalling molecules. In such a scenario activation of the IGF1R and/or IR may promote the selection of the initiated cells that have incorporated mutations in Erk/Akt pathway components (e.g. Ras or PI3K). This could provide a suggestion that treatment with human insulin analogues may initiate the development of mammary tumors with an altered mutational and/or signalling spectrum. More in depth molecular

analysis of the our mouse tumors at the genome and proteome level is needed to further understand the underlying mechanism for the enhanced tumor formation by IGF1 and X10 treatment conditions and their potential role in either enhanced tumor initation and/or progression. While we studied the effect of insulin analogues on mammary gland tumor development, we cannot exclude that insulin analogues with high affinity for the IGF1R may also promote breast cancer progression, either locally or at distant sites, or modulate sensitivity to anticancer drugs. This needs further investigation and our different mouse mammary gland tumor banks that show different expression levels of IGF1R and IR could contribute to this.

Conclusion

The $p53^{R270H/+}$WAPCre mouse model is a sensitive and human relevant model to test the carcinogenic properties of insulin-like molecules, as apparent with insulin X10 and IGF1. Insulin glargine was tested in this study and did not show a significantly decreased tumor latency time compared to insulin NPH, although the MAPK-signalling pathway was upregulated as found for X10 and IGF1. As is the case in humans, rapid conversion of glargine into metabolically active metabolites M1 (and to a lesser extent M2) is likely to be the reason for the low carcinogenic potential of subcutaneous injected glargine. All in all, based on the current tumor model, the data suggest that glargine users are not facing an increased carcinogenic hazard compared to insulin NPH users. Yet future studies in mouse models that lead to more human relevant tumors remain important to fully exclude a role for current clinically relevant insulin analoques in the development and/or progression of human breast cancer.

List of Abbreviations

IGF1, insulin-like growth factor 1; IR, insulin receptor; EMT, Epithelial to Mesenchymal Transition; GEM, Genetically engineered mouse; WAP, Whey Acidic Protein; MPD, Maximal Pharmalogical Dose; NGS, Normal Goat Serum; IF, Immunofluorescence; MG, Mammary Gland; MTLT, Mammary Gland Tumor Latency Time; EC, Endogenous Control

Competing Interest

The authors declare that they have no competing interests.

Contribution statement

Conceived and designed the experiments: BTB, KS, HVS, BVDW, JWVDL. Performed the experiments: BTB, KS, ENS, EK, DS. Analyzed the data: BTB, EK, DS. Wrote the paper: BTB. Reviewed and corrected paper: KS, HVS, DS, EK, ENS, BVDW, JWVDL. All authors approved the final version of this paper.

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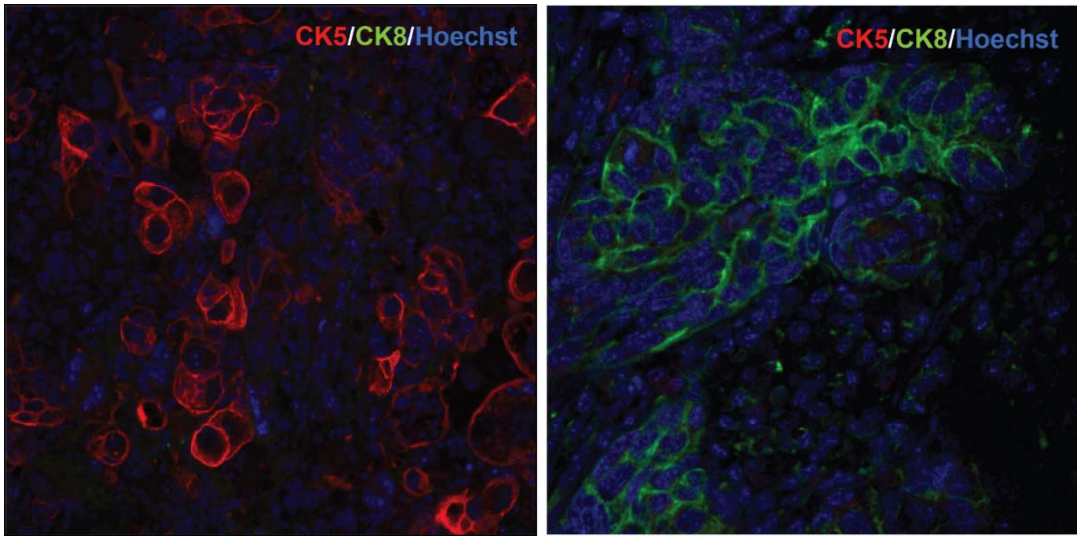
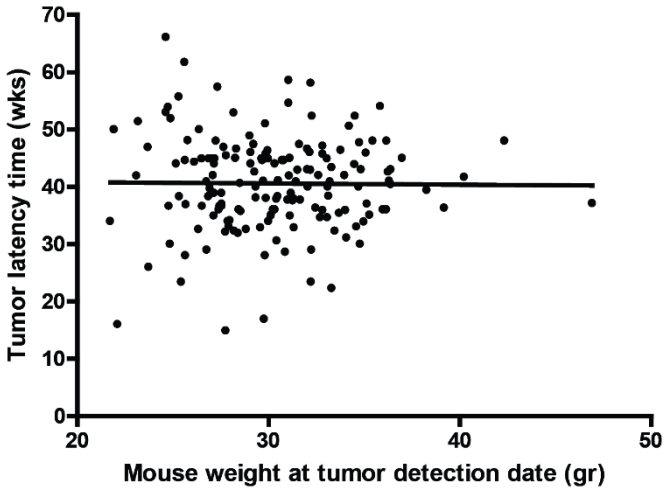
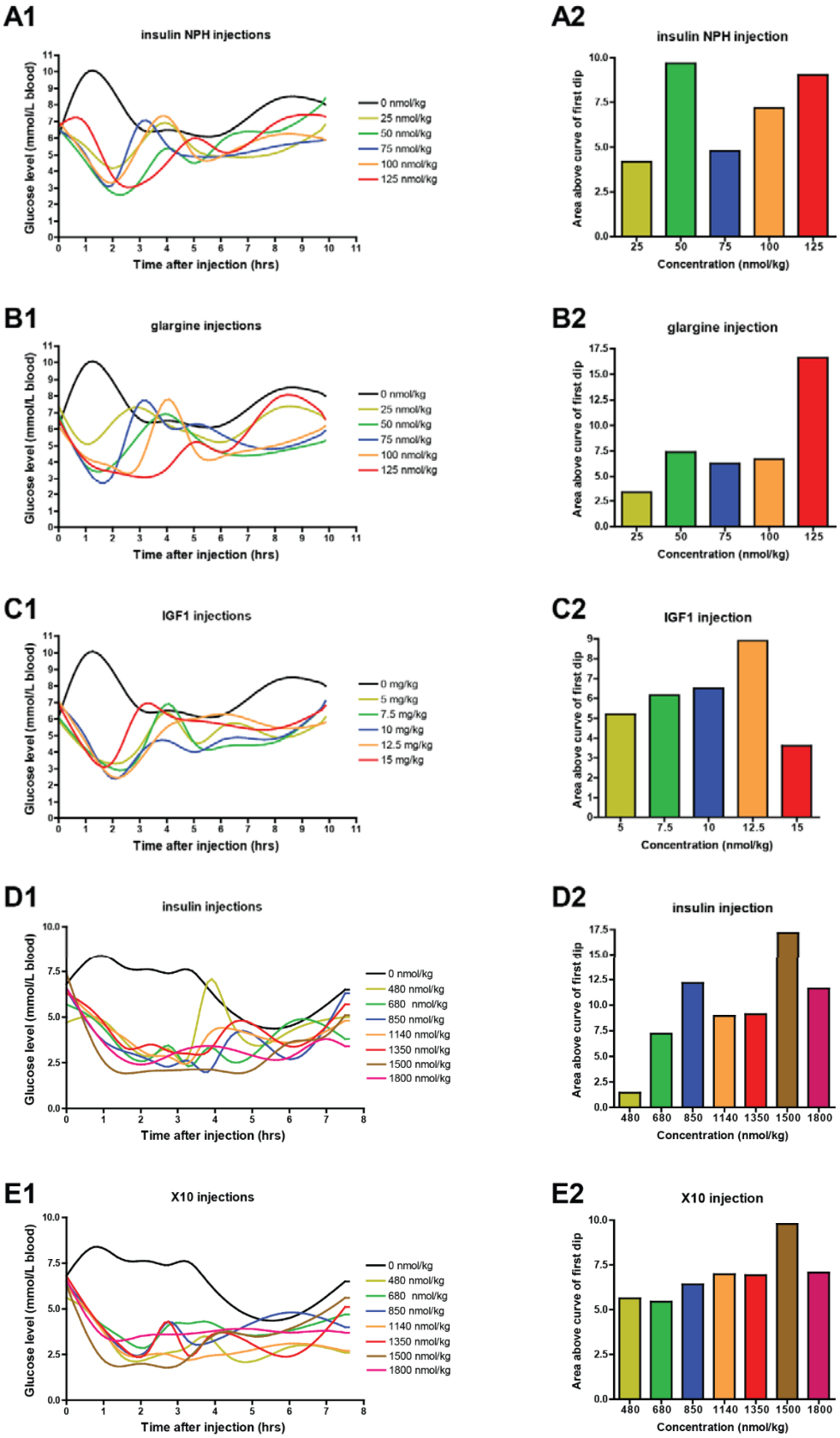
Supplementary figures



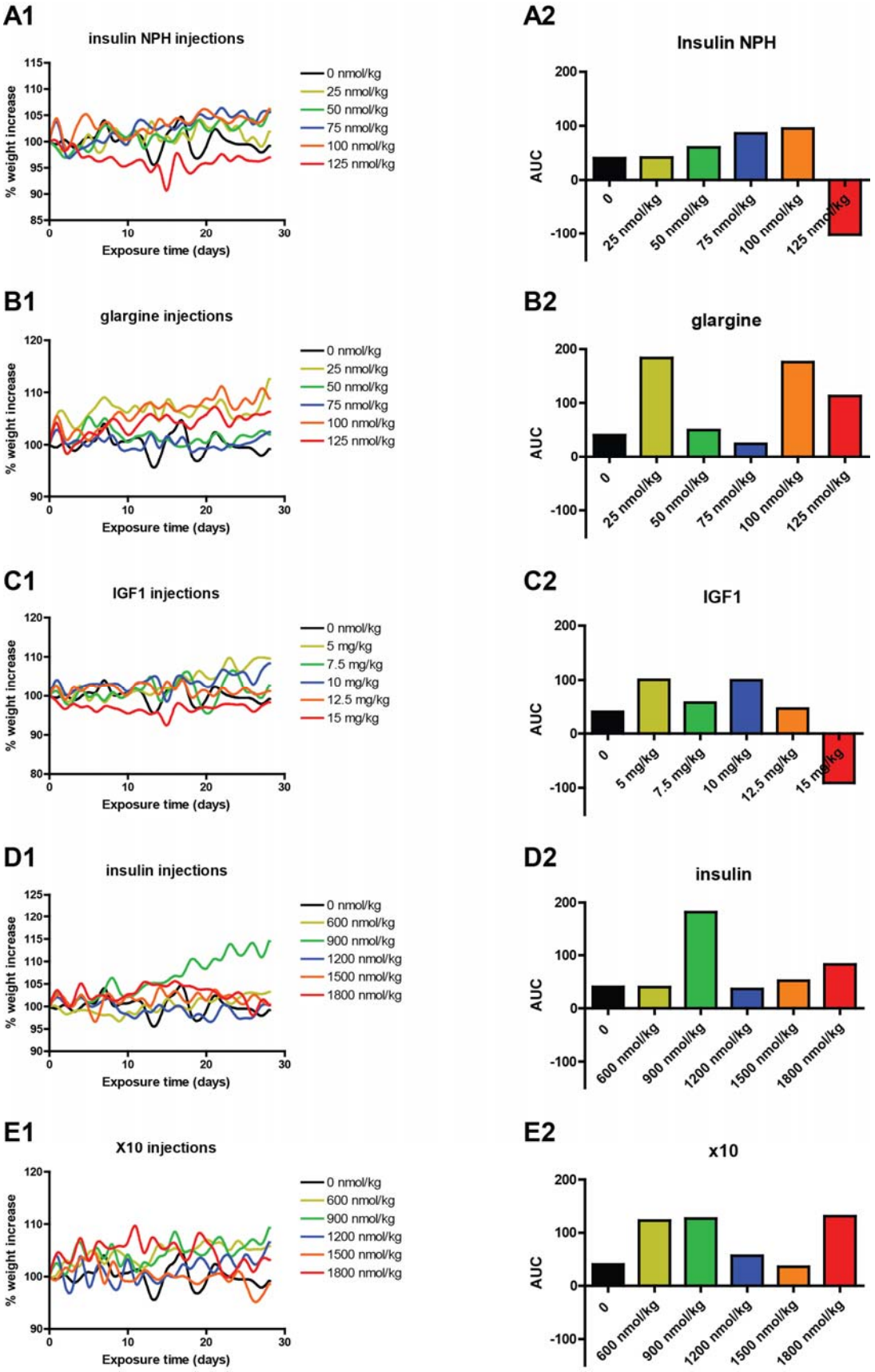
Supplemental Figure 3. EMT tumors can originate from both the luminal as well as myo-epithelial part of the mammary gland. The first IF image is the only EMT tumor that stained positive for CK5, indicating a myo-epithelial origin. The second IF image is a representative example of a tumor that originated from the luminal part of the mammary gland as all cells express CK8 (n=19).



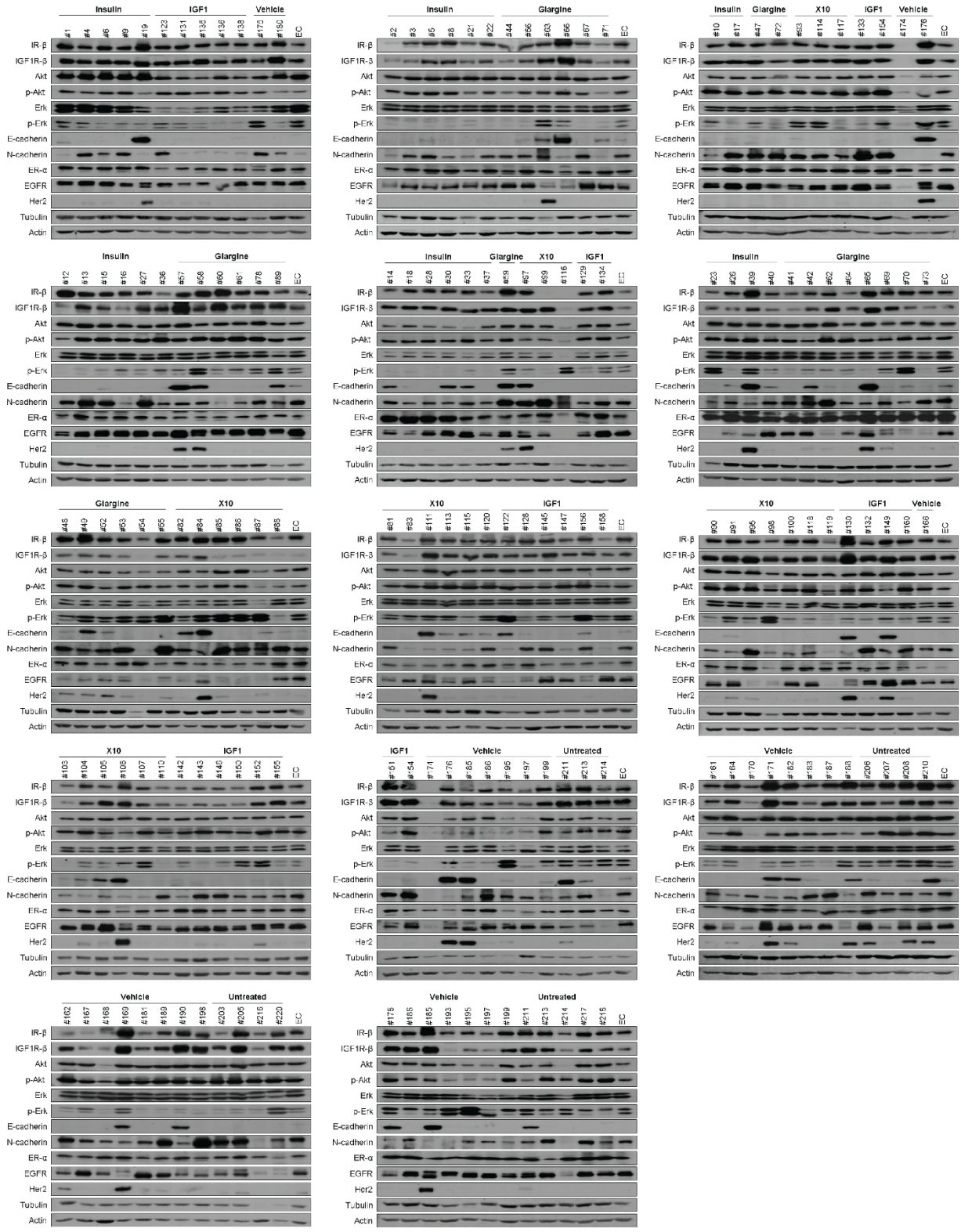
Supplemental Figure 5. No correlation between tumor latencytime and weight of mice at tumor detection date. (p=0.8939, best fit linear slope -0.022)



Supplemental Figure 1. Blood glucose levels measured in p53$^{R270H/+}$WAPCre mice after injections with a concentration range of insulin(-like) molecules. A) insulin NPH injections, B) glargine injection, C) IGF1 injections, D) regular human insulin injections and E) X10 injections. The number 1 graphs represent the blood glucose levels of injected mice over time. The number 2 bar plots represent the area above the curve of the first blood glucose drop (first 3 hours). Each data point represents the average bloodglucose levels of 2 mice.



Supplemental Figure 2. Weight measured in p53$^{R270H/+}$WAPCre mice after injections with a concentration range of insulin(-like) molecules. A) Insulin NPH injections, B) glargine injection, C) IGF1 injections, D) regular human insulin injections and E) X10 injections. The number 1 graphs represent the weight increase in % of injected mice over time. The number 2 bar plots represent the area under the curve of the weight increase plots.



Supplemental Figure 4. Protein expression profiles of all primary mammary gland tumors. Tumor protein levels of critical mammary gland tumor-related receptors (IR, IGF1R, ER, EGFR, Her2) and downstream signalling pathways (Erk, phospho-Erk, Akt, phosphor-Akt) as well as epithelial differentiation markers (N-cadherin and E-cadherin) were determined by quantitative Western blotting of all primary mammary gland tumors (n=148). EC is the endogenous control, a sample that was loaded on every blot to correct for blot specific effects.


Life
Science
Methods

Chapter 6

Tumorigenic insulin analogues promote mammary gland tumor development by increasing glycolysis and promoting biomass production

Highlights

- During tumorigenesis the IRB gene expression levels drop dramatically by which the mitogenic IRA becomes the main insulin receptor in the tumor
- Tumors developed in mice that have been chronically treated with IGF1 show an enhanced mitogenic signalling and migrative gene expression signature compared to tumors from other treatments
- There seems to be a correlation between the mitogenic potential of an insulin analogue and the Warburg effect in tumors of chronically exposed mice
- *Ezh2* and *Hras* mutations are enriched in X10/IGF1 tumors

This chapter is based on a manuscript in preparation:
B. ter Braak, C.L.E. Siezen, J. Lee, P. Rao, C. Voorhoeve, E. Ruppin, J.W. van der Laan, B. van de Water

◀ IN THE PICTURE

Automated 3D spheroid injection system. In vitro models very often rely on 2D cell culture. However, it is thought that 3D systems recapitulate the in vivo situation much better. With the set-up presented in the picture it is possible to inject a cluster of about 1000 cells in a collagen/matrigel environment. We have used this method to study the invasive behaviour of living cells in 3D. These cells have been isolated from tumours discussed in chapter 3.

◀ IN BEELD

Een systeem voor de geautomatiseerde injectie van 3D cel clusters. Ten behoeve van in vitro modellen worden vaak 2D cel cultures gebruikt. Echter, algemeen wordt aangenomen dat 3D modellen de in vivo situatie beter tonen/in beeld brengen. Met het getoonde apparaat is het mogelijk een cluster van ongeveer 1000 cellen in een 3D omgeving te injecteren. Wij hebben deze methode gebruikt om het invasieve karakter van levende cellen te bestuderen.

Abstract

Epidemiological studies suggest that insulin analogues might increase the (breast) cancer risk. In a previous experiment we found that chronic insulin X10 and IGF1 treatment decreased the mammary gland tumor latency time in a $p53^{R270H/+}$WAPCre mouse model. Frequent insulin glargine treatment did not significantly decrease the tumor latency time in this mouse model. Here we performed next generation RNA sequencing on mammary gland tumors to unravel the underlying mechanisms of pro-tumorigenic insulin analogues. We found that chronic X10 and IGF1 treatment resulted in tumors with an increased and sustained proliferative and invasive transcriptomic profile. Furthermore, a Warburg-like effect with increased glycolysis was observed in tumors of the X10/IGF1 groups and to a lesser extend also in glargine induced tumors. A metabolic flux analysis revealed that this enhanced glycolysis programming in X10/IGF1 tumors was associated with increased biomass production programs. Although none of the chronic insulin analogue treatments induced genetic instability or enhanced mutagenesis, mutations in Ezh2 and Hras were enriched in X10/IGF1 treatment tumors. Overall these data suggest that the decreased mammary gland tumor latency time caused by chronic insulin X10 and IGF1 treatment is related to modulation of tumor progression rather than increased tumor initiation.

Keywords: Next generation sequencing, in vivo, Mammary gland tumor, insulin treatment, Glargine, IGF1, Hallmarks of Cancer, Warburg, mutational profiling

Introduction

The administration of exogenous insulin is a common therapy for types I and II diabetes. New insulin-like molecules have small modifications of the insulin molecular structure to improve pharmacokinetic parameters of the original molecule and thereby increase stability and temporal bioavailability. These molecular changes possibly affect the binding affinity towards receptors including the insulin receptor (IR) or the insulin-like growth factor 1 receptor (IGF1R). Consequently, insulin analogues with IGF1R affinity likely promote mitogenesis [191].

Epidemiological studies indicate an increased risk for (breast) cancer in relation to treatment with specific insulin analogues [76] [144]. Yet other studies do not find this association [64] [78] [192]. Epidemiological studies cannot define causal relationships. Therefore many in vitro studies have been performed evaluating possible increased mitogenic properties of insulin analogues [46] [193] [118] [121]. Also here the results have been inconsistent, and further extrapolation from these in vitro systems to the in vivo situation remains difficult [172], (Bronsveld, submitted, 2015). So far in vivo studies that examine the carcinogenic outcome of chronic insulin analogue treatment are limited [175] [46].

Recently we evaluated the tumor promoting capacity of chronic insulin analogue treatment in a humanized breast cancer ($p53^{R270H/+}$WAPCre) mouse model [194]. In this model the WAPCre system ensures mammary gland (MG) specific expression of the heterozygous p53 mutation, which corresponds to a mutational hotspot often found in patients with the *Li Fraumeni* cancer syndrome [195]. Eventually all mice develop spontaneously MG tumors within approximately one year. Chronic treatment with insulin like molecules, IGF1 and the insulin analogue X10, significantly decreased the tumor latency time. Frequent injections with insulin glargine, an insulin analogue currently used by more than seven million diabetic patients worldwide [196], showed a similar trend but the observed tumor latency time decrease was not significant compared to regular insulin [194]. Systematic signaling pathway mapping of all tumors revealed that especially the MAPK/ERK signaling cascade was strongly activated in IGF1- and X10-induced tumors. Although this provides some insight in the alternative signaling wiring in insulin-analogue-related tumors, a systematic evaluation of the genetic modifications and consequently alterations in cellular pathways and network biological differences of insulin analogues related tumors is still obscure.

To gain more insight in the modulation of tumor development and progression by insulin analogues in this study we performed a systematic in depth next generation sequencing (NGS) approach. RNAseq analysis was performed on fifty insulin analogue-induced MG tumors (control, insulin, IGF1, X10 and glargine treatment). Overall genetic modifications were determined on a tumor level. NGS transcriptome analysis did shed light on the specific tumor development and progression in relation to insulin analogue type. For this we specifically evaluated the alternative modulation of the hallmarks of cancer [197] to detect treatment specific tumor features.

Materials and Methods

Chronic in vivo insulin analogue treatment

Previously we have reported the effect of insulin analogues on tumor development [194]. In short, for the chronic exposure experiment two hundred (40 mice per treatment), 8 week old female $p53^{R270H/+}$WAPCre mice were obtained from an in house breeding project. The point mutation in the tumor suppressor p53 gene corresponds to the Li Fraumeni Cancer syndrome mutational hotspot (R273H) in humans. Every other day these mice have been injected (subcutaneously) with either vehicle, insulin, glargine, X10 or IGF1 till tumor development. Once the tumors reached a size of 1 cm^3 and 24 hours after their last injection the mice were sacrificed. Tumors and other tissues were isolated. ¼ of the tumor was stored in RNALater (Ambion, Austin, Texas) at 4 degrees Celsius for RNA isolation. A miRNA isolation kit (Macherey Nagel, Germany) was used to isolate and purify small and large RNA molecules in one fraction.

Single insulin analogue treatment; an animal experiment

For the single insulin analogue exposure experiment forty (4 mice per treatment/time point) female 12-week-old inbred FVB/NRj mice were obtained from Janvier, rodent research models, France. Housing and experiments were performed according to the Dutch guidelines for the care and use of laboratory animals (UL-DEC-14020). Mice received a single subcutaneous injection with either vehicle, insulin, glargine, X10 or IGF1. One or six hours after the injection the mice were sacrificed, blood was collected (mini collect, Greiner/Omnilabo) and MGs were stored in RNALater (Ambion, USA) at 4 degrees C for RNA isolation. For this a Nucleospin RNA isolation kit (Macherey Nagel, Germany) was used. For further technical details we refer to our previous publication [194].

In vitro stimulation experiments

MCF7 human breast cancer cells with an overexpression of the IGF1R and a stable knockdown of the INSR (MCF7 IGF1R) have been seeded at 60% confluence and starved for 2 days in 5% CDFBS (Hyclone, USA) containing RPMI 1640 (Gibco, USA) medium. Cells were stimulated (with 10 nM compound) for 1 or 6 hours after which RNA was isolated using NucleoSpin® miRNA isolation kit (Machery Nagel, Düren, Germany). Stimulations included: insulin NPH (Insuman Basal, Sanofi Aventis), insulin glargine (Lantus, Sanofi Aventis), insulin X10 (AspB10, Novo Nordisk) and IGF1 (Increlex, Ipsen). For a more technical details we refer to our previous publication [194].

Next generation sequencing (NGS), gene expression analysis

The quality and integrity of the RNA samples were analysed using the bioanalyzer with a RNA nanochip. The Ion Total RNA-Seq kit was used to process the samples. Samples were Poly-A selected prior to library preparation. This library preparation included the cDNA synthesis and purification steps with the Ion Total RNA-Seq kit v2 (Life Technologies, UK) according to manufacturer's instructions. The Ion PI Template OT2 200 Kit v3 and Ion Sequencing 200 kit v3 (both, Life Technologies, UK) were used according to manufactures instructions for sequencing libraries on the PI chip. Sequence runs were performed on the Ion Proton Sequencer (at ServiceXS, Leiden). PI chip analysis, base calling and quality checks were performed using the Torrent Server Suite. On average 40 million reads per sample were sequenced with an average read length of 100 base pairs. No additional trimming or filtering of reads was performed before processing. Reads were aligned to mouse genome build GRCm38 - Ensembl using Tophat2 (Version 2.0.10). Reads which could not be aligned using TopHat2 were aligned in an additional step, using Bowtie2 (Version 2-2.10) in the local, very sensitive mode. Tophat2- and Bowtie2-aligned reads were merged into a single .bam file for each sample before further analysis.

Gene expression was quantified using HTSeq-Count (Version 0.6.1), using the default options. Differential gene expression was analyzed for compound versus vehicle treatment and was performed using DESeq2 (Version1.2.10). For this analysis, genes with a read count of < 50 reads

across samples (average of <1 read per sample) were filtered out before the analysis. For the estimation of individual exon expression analysis a RPKM table was generated with the read counts normalized to library size and gene length (using DEXSeq version 1.8.0). For the mutational profiling the reads (unfiltered and untrimmed) were aligned to mouse genome build GRCm38.73- DNA primary assembly using TMAP within the Torrent Suite version 4.0.2. Variant calling was performed using the Torrent Suite Variant Caller version 4.0-r72612 with the settings tuned for the detection of somatic mutations at a low stringency level. The reference genome used was the same as that used for read alignment - GRCm38.73. SnpEff version 3.6c was used to filter and annotate the mutations. The list of mutations was filtered to include only exonic mutations with a quality score higher than 250. Several mutations are found in the exact same position in all tumor samples, probably strain-specific single nucleotide polymorphisms (SNPs). Known SNPs in coding regions for the mouse strain FVB/NJ (the strain most closely related to the $^{p53R270H+/-}$WAPCre) were downloaded from the Mouse Genome Informatics database and these mutations were discarded from the list. Mouse homologs of the list of human tumor driver genes [198] were used to define the most clinically relevant mutations.

Phenotypic prediction based on transcriptomic data

To predict the phenotypic characteristics of the treatments of different insulin-analogues using their transcriptomics, we constructed a support vector machine (SVM) classifier. The procedure is described by Yizhak et al, in which the genes have been identified in 52 human breast cancer cell lines that are important for migration and proliferation based on their respective expression levels and functional bio-assays [199]. We first identified the transcriptomic signatures of migration and proliferation based on the 52 breast cancer cell line data. We then applied them to the orthologs of the mouse transcriptomics data to predict the migratory and proliferative potential of 50 mouse mammary tumor samples using LIBSVM [200].

Metabolic flux analysis

We estimated the metabolic fluxes that are most consistent with the transcriptomics data using a computational framework called iMAT [201]. iMAT integrates the transcriptomics, as 'soft' constraints, by ternary partitioning the expression to lowly (-1), mediocrely (0) and highly (1) expressed genes. iMAT then attempts to collect the metabolic states that best correspond to these cues, which constructs a mixed integer linear programming (MILP) problem. We applied iMAT to the human genome-scale metabolic model Recon1 [202] with a standard medium condition (DMEM) in a condition-specific manner for the 5 different insulin analogues treatments. With the predicted metabolic fluxes by iMAT, we performed a pathway enrichment analysis of differentially activated metabolic reactions in X10/IGF1 treated cells to the remainder conditions (insulin/glargine/vehicle). We selected the metabolic pathways whose reactions are

significantly enriched in the up-/down- regulated group using hypergeometric test followed by multiple hypotheses correction with the false discovery rate (FDR) 0.05. The predicted biomass production rate does not involve standard deviation because we focused on the metabolic states where the biomass production rate is optimized (thus single-valued).

Statistical analysis

For the statistical analysis Graphpad Prism version 5.01 software was used. All standard error bars in the graphs represent standard deviations. Multi-experiment viewer (MeV version 4.8.1) was used for the hierarchical clustering analysis.

Results

Genetic profiling of insulin analogue induced mammary gland tumors might reveal cellular processes that have initiated the decreased tumor latency

Previously we chronically treated $p53^{R270H/+}$WAPCre mice with insulin and insulin analogues (Figure 1A) and found that MG tumor latency time in X10 and IGF1 treatment groups was significantly decreased (Figure 1B). Tumors in the glargine treated mice also developed earlier compared to vehicle and insulin treated animals, but this trend was not significant [194]. To further investigate this we evaluated receptor gene expression levels. We found that gene expression levels of the insulin receptor (both the A and B isoform; *ira, irb* respectively) and insulin-like growth factor 1 receptor (*igf1r*) are significantly up regulated in pre-neoplastic MG tissue of old $p53^{R270H/+}$WAPCre mice compared to healthy 8 week old MGs (Figure 1C). Also *ira* and *igf1r* levels were slightly up regulated in MG tumors. Interestingly, we found a significant and seven-fold down regulation of the B-isoform of the insulin receptor in MG tumor tissue compared to normal MG tissue. This effect was not treatment specific (Figure 1C right). Levels of *igf1r* were only decreased after chronic treatment with IGF1. These data are indicative for an involvement of INSR/IGF1R signalling in tumor development and/or progression. However, the different receptor distributions cannot explain the differences in tumor latency time, since there was no treatment-related effect.

To gain more insight in overall differences between various insulin analogues in tumor development and progression, we performed RNAseq NGS of 50 tumors, 10 tumors per treatment group. We reasoned that transcriptome analysis would shed light on the differences in tumor latency time by chronic insulin analogue treatment. From the 40 tumors per treatment group, we selected the 10 EMT-like tumors per treatment condition as well as those tumors for which the tumor latency time was closest to the median latency time of that entire specific treatment group. In Figure 1D a correlation analysis shows the sample-to-sample distance. All transcriptomics data was used to define the correlation within and between the different treatment groups.

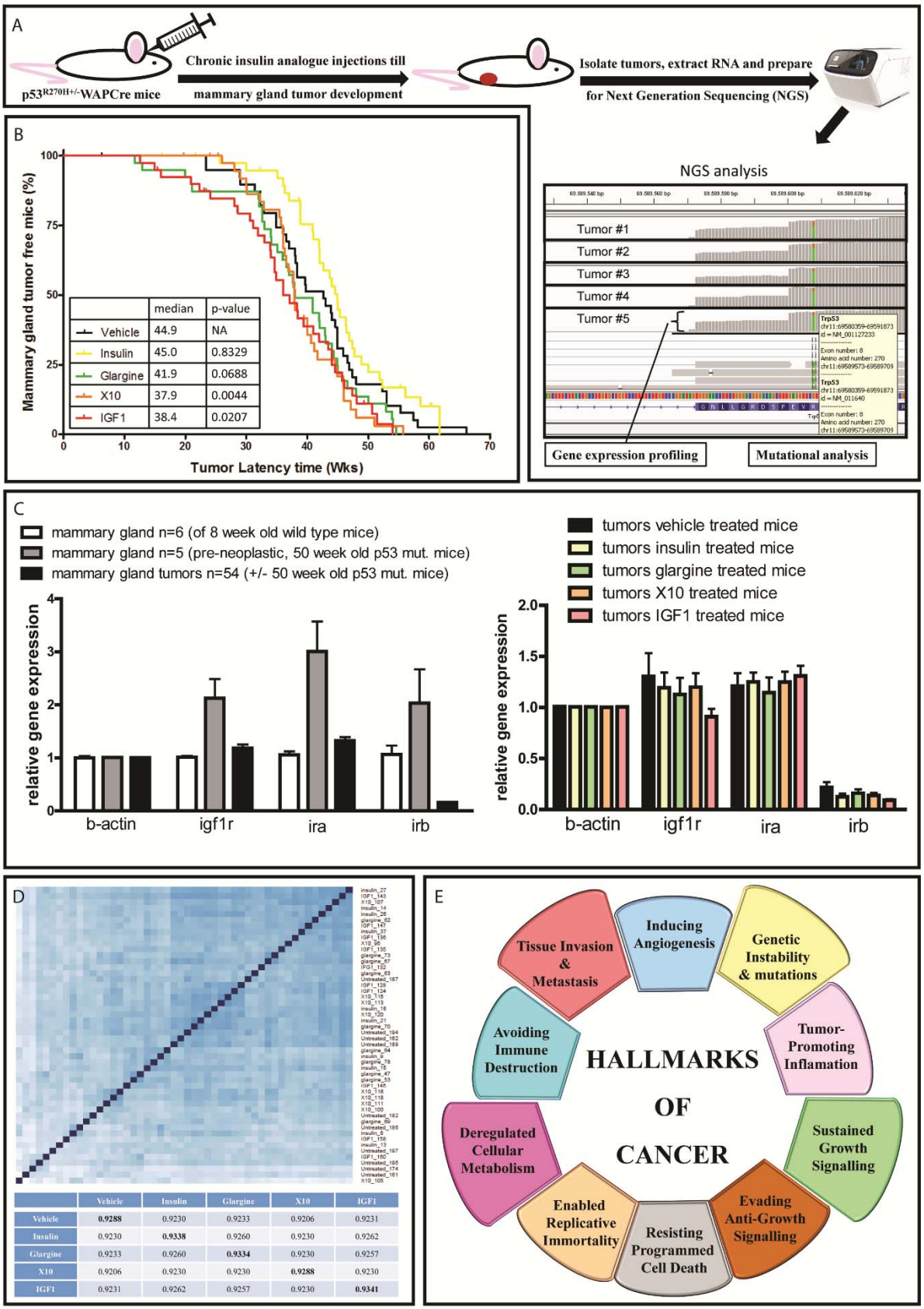


Figure 9. Experimental overview. A) Overview of the chronic insulin analogue exposure experiment. B) The Kaplan Mayer MG gland tumor free mice plots with the median tumor latency per treatment group. C) the IGF1R, IRA, IRB gene expression levels in MG tissue of young mice, old mice and in MG tumors (first graph), second plot shows the receptor gene expression distribution in the MG tumor tissue of mice chronically exposed to different insulin

analogues. D) Heat map of the next generation sequencing data showing hierarchical sample clustering by sample-to-sample distance, the lower table shows the Spearman rank correlation coefficients within the treatment groups (bold) and the coefficient between the different treatment groups (averaged per condition). E) The hallmarks of cancer with the features highlighted that we will discuss in in view of the chronic insulin analogue exposure experiment. (Adapted from Hanahan and Weinberg, 2011)

In spite of the relatively high variation in this in vivo experiment, there was a higher correlation within each treatment group than between the different treatment groups. This suggests the existence of treatment specific response in each treatment conditions. To define these treatment specific responses we focussed on four particular hallmarks of cancer (Figure 1E), that might have been involved in the decreased tumor latency, either by tumor initiation or progression. These pathways were selected in close relation to the signalling pathways downstream of IR and IGF1R and included: i) sustained growth signalling; ii) tissue invasion and metastasis; iii) deregulated cellular metabolism; and iv) genetic instability and mutations.

X10- and IGF1-derived tumors demonstrate 'sustained growth signaling'.

Proliferative signaling is normally a highly regulated process. In a tumor the replicative cell homeostasis is deregulated causing sustained growth signaling [197]. Since mice were chronically exposed to insulin analogues that activate the INSR/IGF1R, we postulated that the decreased tumor latency time (after X10/IGF1 stimulation) would be a direct result of an up-regulated INSR or IGF1R signaling pathway. We selected mouse orthologous of human genes (~60 genes) that are involved in INSR and IGF1R signaling according to Ingenuity Pathway Analysis (IPA) (Figure 2A). A hierarchical clustering analysis was performed for all 50 MG tumors on the relative expression levels of these genes (Figure 2B). Two clusters could be defined. In cluster 1 tumors from low mitogenic treatments are enriched, whereas cluster 2 predominantly consists of the X10/IGF1 treated tumors. These two different cluster could not be linked to specific signaling pathways (e.g. PI3K, MAPK, JAK signaling cascade).

To predict the proliferative potential of all the individual tumors, we next built an SVM classifier using transcriptomic signatures associated with proliferation (Figure 2Ci). No significant effect was observed; however, a clear trend for the tumors in the X10 and IGF1 treatment groups to have an increased proliferative potential could be seen. As a positive control for the SVM simulation, similarly we ran the SMV simulation on our earlier micro-array data of the MCF7-IGF1R cells stimulated with the different insulin analogues. We observed that 1 hour after glargine, X10 and IGF1 stimulation a strong and significant proliferative genetic signature could be detected.

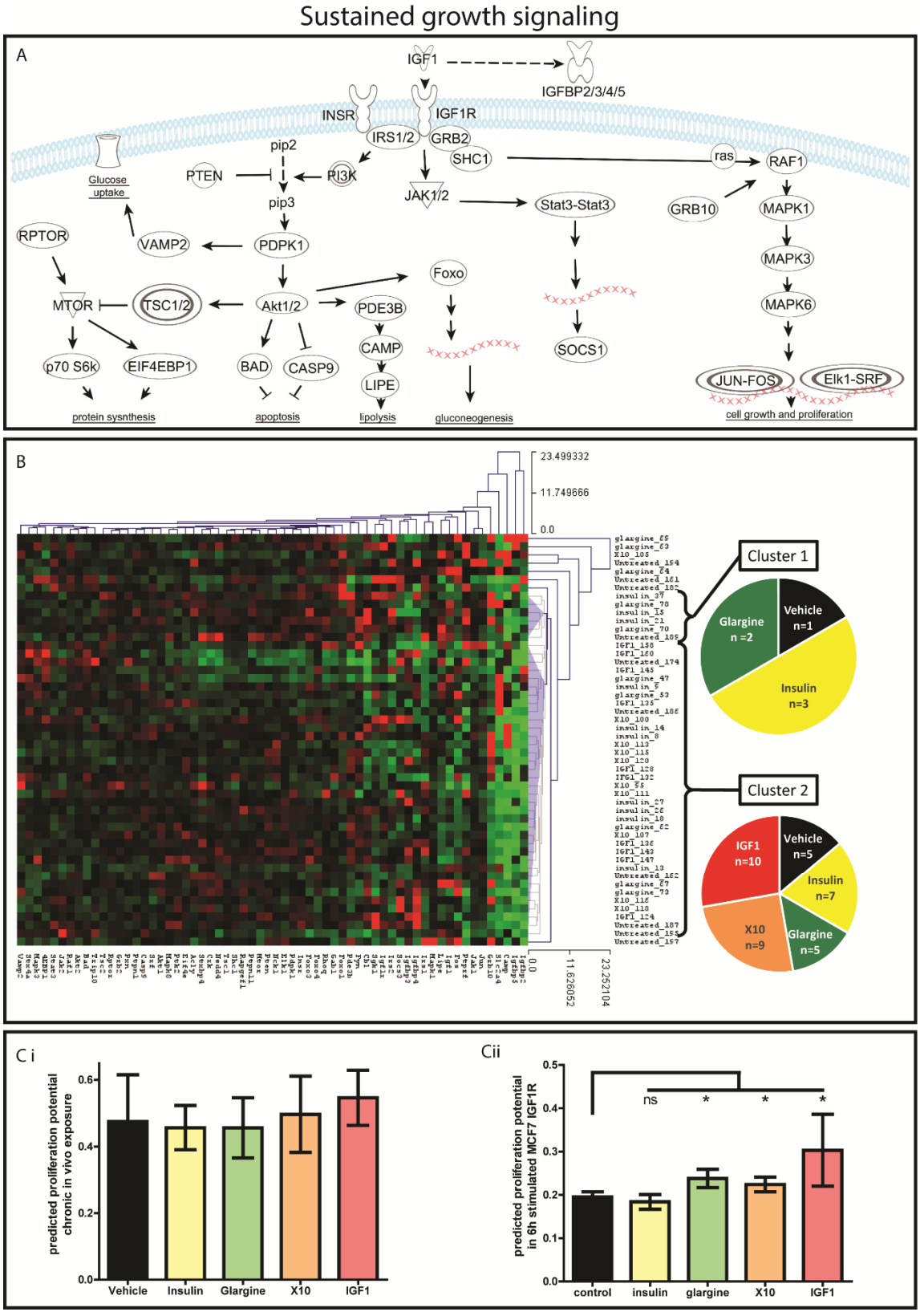


Figure 10. Sustained growth signaling in mammary gland tumor tissue of chronically i.a. exposed mice. A) The INSR/IGF1R signaling pathway with receptors, downstream targets and the biological effect. B) Hierarchical clustering (Euclidian distance) of the INSR/IGF1R specific gene expression per MG tumor. The pie-diagrams show the distribution of the different treatments in the two clusters. C) Bar graph of SVM simulation on the predicted proliferation potential per treatment of MG tumors of the chronically exposed mice (Ci) and human breast cancer cells (MCF7 IGF1R) exposed for 1h to the indicated i.a (Cii)

Chronic X10 and IGF1 treatment induce tumors with a more mesenchymal phenotype and a higher migration potential

Next we assessed hallmark "Tissue invasion and metastasis". Again we selected genes that are directly associated with the epithelial to mesenchymal transition (EMT) or the mesenchymal to epithelial transition (MET) according to IPA. The gene expression of the mouse orthologues of these genes is presented in an unsupervised hierarchical clustering (Figure 3A). Two clear gene groups were observed: group A containing genes associated with epithelial phenotype (*Elf5, Serpinb5, Cdh1, Grhl2, Elf3, Wnt4*), and group B containing twelve genes associated with mesenchymal cells (*Pdgfrb, Six1, Snai2, Tcf4, Zeb1, Klf8, Wnt5a, Snail, Vim, Twist1, Cdh2*). From this hierarchical clustering three separate treatment clusters could be discriminated. Cluster 1 consists of tumors that show high expression of epithelial and low expression of mesenchymal associated genes, three out of six tumors originate from the vehicle treated animals. Cluster 2 shows moderate expression levels of epithelial and mesenchymal associated genes, interestingly the majority of these tumors were from the glargine treatment condition. Cluster 3 consists of tumors that have a low expression of epithelial and high expression of mesenchymal markers. IGF1 treatments were not present at all in cluster 1 and 2. While IGF1 and X10 treatment groups were overrepresented in cluster 3. This suggests that IGF1R-mediated signaling is a driver of tumors with an EMT phenotype. We substantiated the phenotypes for the three different clusters (Figure 3B). The tumors in cluster 1 predominantly expressed epithelial cells that contain many E-cadherin positive cells. Tumors of the cluster 2 and 3 phenotype were characterized as EMT tumors predominantly consisting of mesenchymal cells, lack of E-cadherin staining and clear smooth muscle actin (SMA) staining (data not shown) [194].

To link the transcriptomics data further to the phenotype we performed a SVM simulation to derive predictions for the cell migratory potential of each tumor (Figure 3Ci). Overall no significant difference in the migration potential was observed for the different treatment groups. To evaluate whether IGF1 signaling itself is a strong activator in vitro, we also applied our SVM classifier to our previous MCF7-IGF1R transcriptomics data exposed to the different insulin analogues (Figure 3Cii). IGF1, X10 and glargine caused a significant increase in the migration potential. Altogether these data suggest that chronic exposure to X10, and especially IGF1 induces tumors with a more a mesenchymal phenotype that are possibly more aggressive in terms of their migratory potential.

Mice receiving a chronic X10 and IGF1 treatment develop tumors with a higher Warburg potential

Through the activation of the INSR, insulin and insulin analogues directly affect cell metabolism. Therefore we wondered whether the chronic treatment with insulin analogues induce tumors with a deregulated cellular metabolism or a higher Warburg potential.

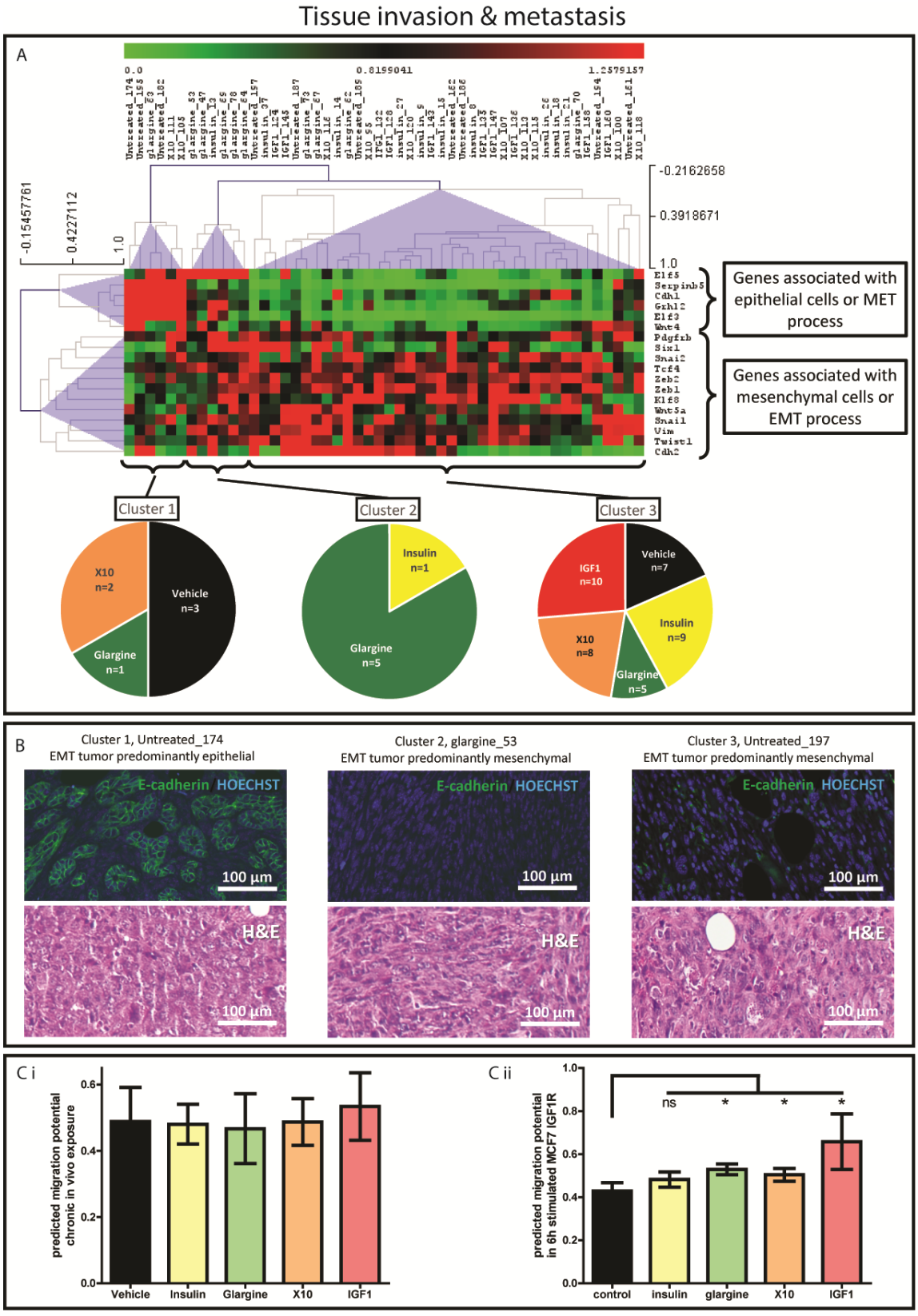


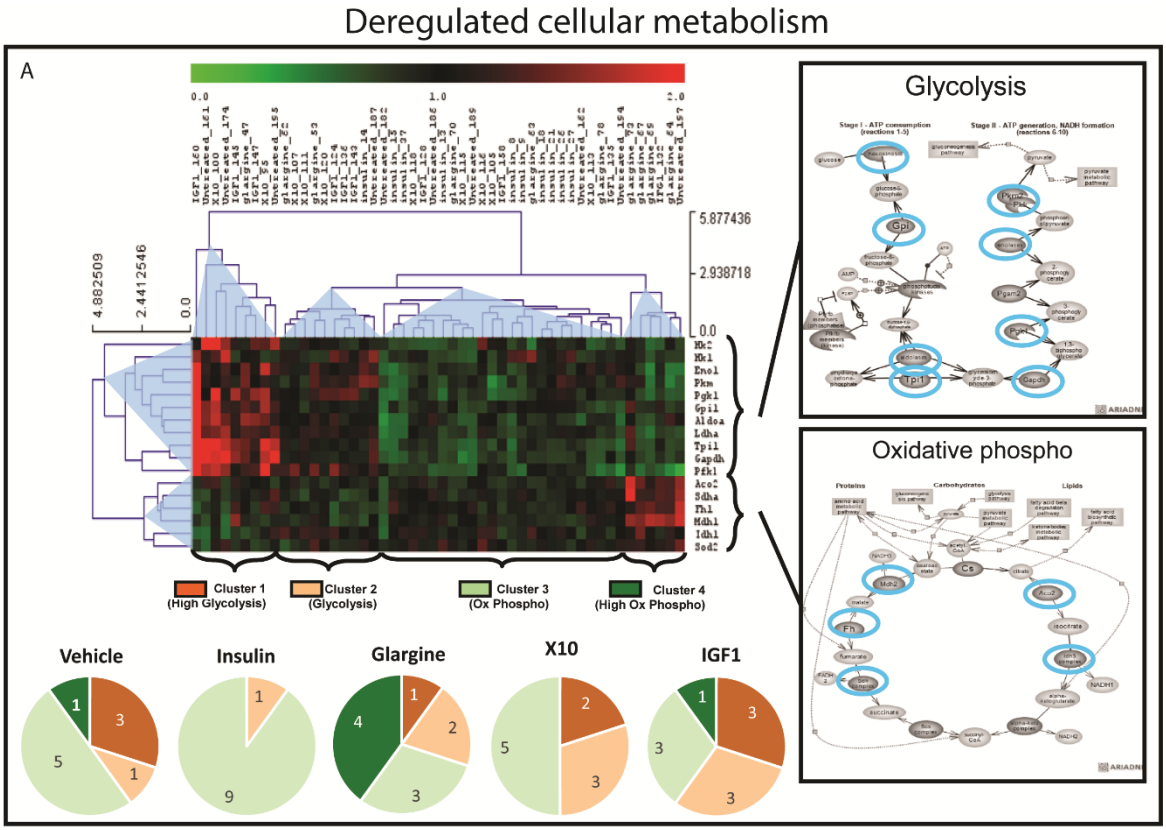
Figure 11. Tissue invasion and metastasis in mammary gland tumor tissue of chronically i.a. exposed mice. A) Hierarchical clustering (Pearson correlation) of genes involved in EMT or MET per MG tumor. The pie-diagrams show the distribution of the different treatments in the three clusters. B) shows the E-cadherin/HOECHST immuno fluorescent H&E pathology slides of 3 example tumors showing epithelial or mesenchymal characteristics C) Bar graph of SVM simulation on the predicted migration potential per treatment of MG tumors of the chronically exposed mice (Ci) and human breast cancer cells (MCF7 IGF1R) exposed for 1h to the indicated i.a (Cii)

To test this we defined genes directly involved in glycolysis and in oxidative phosphorylation according to IPA. An unsupervised hierarchical clustering of the expression profiles of each tumor revealed two very clear gene groups (Figure 4A). Strikingly, the first gene group consists of eleven genes that are all directly involved in glycolysis; all the genes in the second cluster are involved in oxidative phosphorylation. Four clear tumor clusters could be detected: cluster 1 with high expression of glycolysis genes and low expression of oxidative phosphorylation genes; cluster 2 with moderate glycolysis; cluster 3 with little glycolysis and moderate oxidative phosphorylation; and cluster 4 with high oxidative phosphorylation and little glycolysis. For the insulin group almost all tumors were related to cluster 3 (little glycolysis and moderate oxidative phosphorylation: 90%). For glargine-related tumors 70% relied on oxidative phosphorylation. IGF1 and X10 were relatively more dependent on glycolysis (60% and 50%, respectively) comparable to vehicle control. This suggests that chronic insulin signaling drives tumors in a more oxidative phosphorylation modes.

Since IGF1 and X10 were mostly related to glycolysis we performed an independent bioinformatics analysis on the activity of various metabolic processes. This revealed several significantly down- and up regulated metabolic programs in IGF1/X10-derived tumors compared to the vehicle/insulin/glargine tumors (Figure 4B). Importantly, as expected oxidative phosphorylation was significantly down regulated in the IGF1/X10 group ($p=0.000107$) while glycolysis was significantly upregulated. Again, this analysis was also performed on the microarray data of MCF7-IGF1R cells exposed to the different insulin analogues. Similarly to the in vivo data IGF1 and X10 treatment significantly down regulated the citric acid cycle as well as oxidative phosphorylation (both $p<0.0005$) and up regulated glycolysis ($p<0.00001$) (Figure 4C). These data suggest that both IGF1 and X10 promote a Warburg effect in mammary gland tumors and human tumor cell lines.

Biomass production rate is highly up regulated in tumors of chronic X10/IGF1 treated mice

Tumor mice receiving a chronic X10 and IGF1 treatment showed a glycolytic activity (figure 4) associated with decreased tumor latency time (Figure 5A), we evaluated whether this difference in metabolic capacity is related to enhanced growth. To test this hypothesis we predicted the rate of the accumulation of biomass in all these different tumors based on the gene expression profiles of biomass producing metabolic processes. In Figure 5B a bar graph of these results is presented. A highly increased mammary gland tumor biomass production rate was observed in chronic X10 and IGF1 treated tumors. This finding is in agreement with the observed increased proliferative potential in the X10/IGF1 tumors. These results strongly suggest that the decreased tumor latency time by X10 and IGF1 treatment was caused by enhancing the tumor development rather than interfering with the tumor initiation.



B

Metabolic pathways downregulated in X10/IGF1 treatment groups	p-value
Riboflavin Metabolism	1E-300
Transport, Extracellular	1E-300
Folate Metabolism	1E-300
Transport, Mitochondrial	8.1E-10
Oxidative Phosphorylation	**1.4E-07**
Nucleotides	*0.0001*

Metabolic pathways upregulated in X10/IGF1 treatment groups	p-value
Glutamate metabolism	1E-300
Heme Degradation	1E-300
Hyaluronan Metabolism	1E-300
Riboflavin Metabolism	1E-300
Salvage Pathway	1E-300
Taurine and hypotaurine metabolism	1E-300

C

Metabolic pathways downregulated after X10/IGF1 stimulation in MCF7	p-value
Fatty acid Metabolism	1E-300
Keratan sulfate degradation	1E-300
Nucleic acid degradation	1E-300
ROS Detoxification	1E-300
Sphingolipid Metabolism	2.5E-13
Valine, Leucine, Isoleucine Met.	2.6E-10
N-Glycan Degradation	3.1E-06
Chondroitin sulfate degradation	4.8E-06
Glycine, Serine, Threonine Met.	7.8E-06
Citric Acid Cycle	**1.0E-05**
Heparan sulfate degradation	2.7E-05
Bile Acid Biosynthesis	0.0002
Oxidative Phosphorylation	**0.0002**
Folate Metabolism	*0.0008*

Metabolic pathways upregulated after X10/IGF1 stimulation in MCF7	p-value
D-arg and D-orn metabolism	1E-300
Keratan sulfate biosynthesis	1E-300
Salvage Pathway	1E-300
Tyr, Phe, Trp Biosynthesis	1E-300
Transport, Extracellular	1.1E-16
Steroid Metabolism	3.3E-16
Tyrosine metabolism	6.2E-09
Transport, Mitochondrial	1.7E-06
Glycolysis/Gluconeogenesis	**5.4E-06**
Blood Group Biosynthesis	7.1E-06
Cholesterol Metabolism	6.1E-05
CYP Metabolism	0.0001
Amino group metabolism	0.0002
IMP Biosynthesis	0.0002

Figure 12. Warburg effect in mammary gland tumor tissue of chronically insulin analogue treated mice. A) Hierarchical clustering (Pearson correlation) of genes involved in glycolysis or oxidative phosphorylation per MG tumor. The pie-diagrams show the distribution of the different clusters per treatment group. B) Table with the metabolic pathways that were significantly down- or up- regulated in the X10/IGF1 treatment groups compared to the vehicle, insulin and glargine treatment groups. C) Table with the metabolic pathways that were significantly down- or up- regulated after X10/IGF1 exposure compared to vehicle, insulin and glargine treatment in the MCF7 IGF1R model.

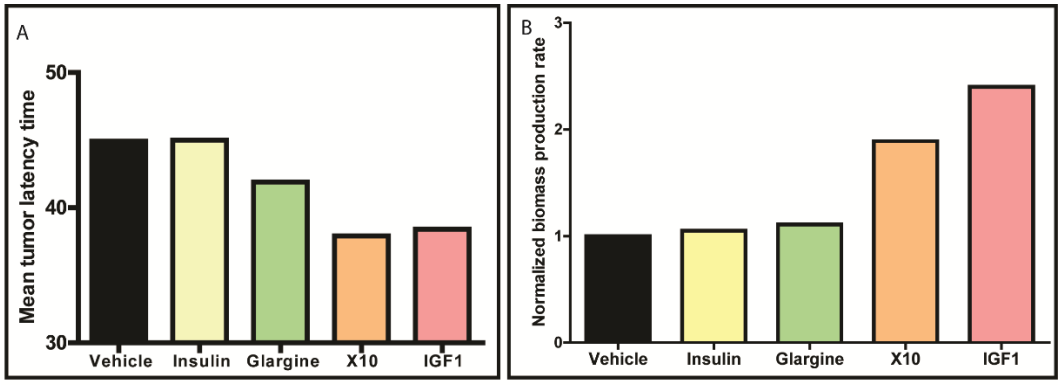


Figure 5. Biomass production rate is increased in tumors of chronically X10/IGF1 treated mice and could possible explain the decreased tumor latency time in these treatments. A) Bar plot of the mean tumor latency time in weeks per chronic treatment. B) Bar plot of the normalized biomass production rate per treatment.

Ezh2 and Hras mutations are enriched in X10/IGF1 tumors.

Since IGF1 and X10 promote cell proliferation, this could lead to a manifestation of mutations and consequently modulation of tumor development and progression. To evaluate if chronic IGF1 or X10 treatment affects the number of mutations a mutational analysis was performed on all tumors based on the 40 million 100 base pair reads for each tumor. The number of different mutations was ~3000 mutations per tumor and no significant difference between treatment groups could be detected. Also, there was no correlation between the number of mutations per tumor and the tumor latency time (best fit correlation values, slope:0.00001207, r^2:0.0000099, p-value: 0.9827) (Figure 6A).

Next we focused on specific clinically relevant mutations that are part of the ~140 known human tumors drivers according to Vogelstein and colleagues [198]. In the fifty mouse tumors that we sequenced, 102 of these tumor drivers were mutated. On average each tumor had about 35 of these tumor driver mutations and again it seems that the chronic treatment did not affect the overall number of tumor driver mutations per tumor (Figure 6B). Also for the tumor driver mutations there was no correlation between number of mutations and latency time of each tumor. Also no treatment specific effects could be detected for the average number of point mutations, the average number of frame shifts, the average number of start CODON insertions and the average number of stop CODON insertions (Supplemental Figure 1).

We next determined the mutations are overrepresented (>50%) in the X10/IGF1 treatments (Figure 6C). All mutations are involved in cancer development and/or progression [198] [203]. Although no specific core pathway links these individual mutations, strikingly Ezh2 and Hras were strongly overrepresented in IGF1/X10 treated tumors. In a similar way we looked at specific mutations that have been overrepresented in insulin analogue treatment altogether and highly underrepresented in the vehicle condition. No general mechanism was identified that could explain the enrichment of these mutations in the treatment groups. However, a set of five

mutations was not at all detected in vehicle control, yet specifically affected in the insulin treatment group. Overall these data suggest that the IGF1/X10 enhanced tumor formation is associated with several key candidate cancer drivers that could contribute including Ezh2 and Hras, which are known drivers in human breast cancer [204] [205].

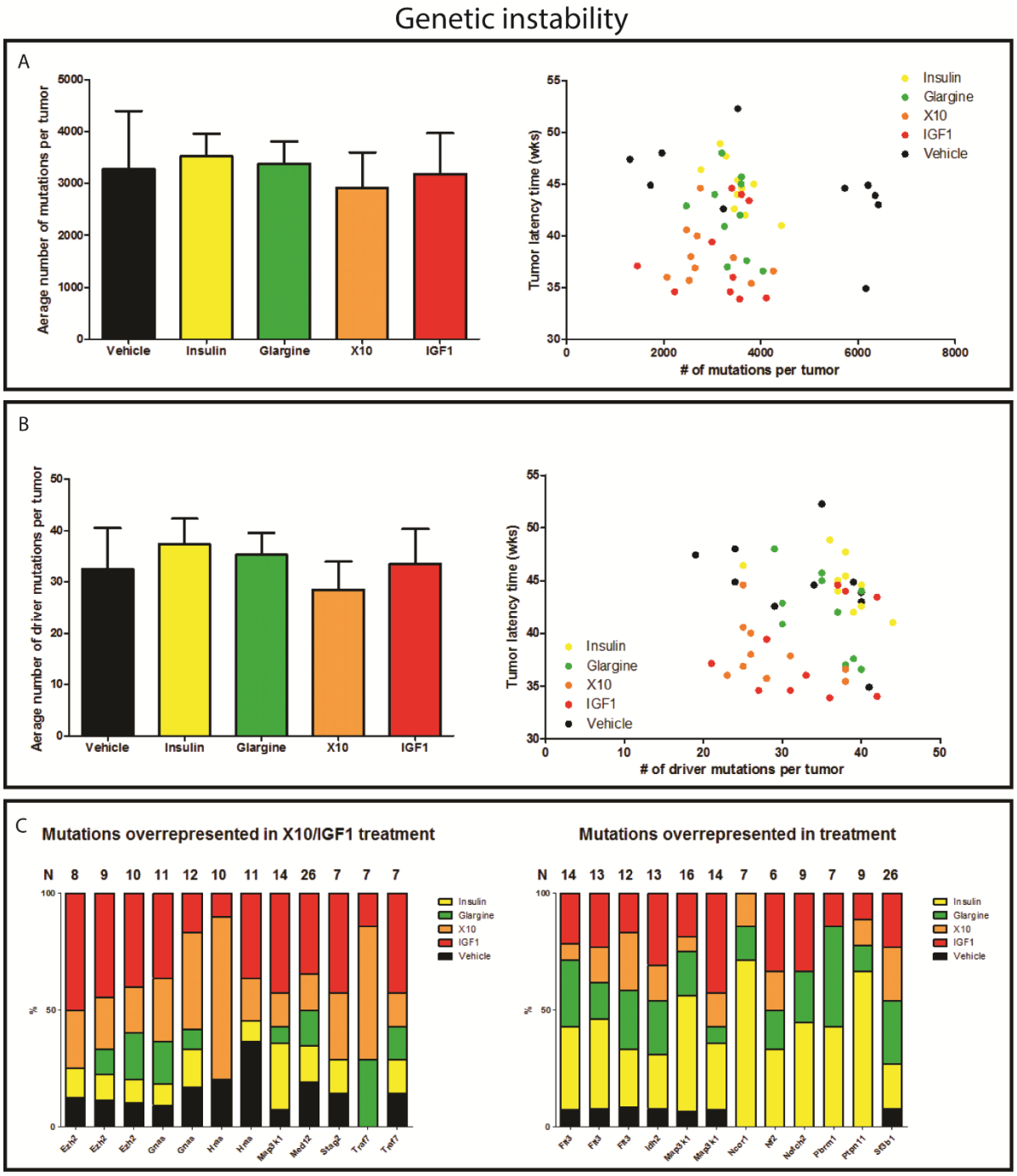


Figure 6. Genetic instability in mammary gland tumor tissue of chronically insulin analogue exposed mice. A) The bar plot shows the average number of mutations per MG tumor for all chronic treatments, the dot plot indicates that there is no correlation between the number of mutations of a specific tumor and tumor latency time. B) Same as in A, but here we focus on clinically relevant human tumor driver mutations. C) Some specific tumor driver mutations are featured in these bar plots. The first barplot represents the mutations that are enriched in the

X10/IGF1 treatment groups, the second barplot the mutations are highlighted that are underrepresented in the vehicle treatment group. The N shows the number of tumors in which this specific mutation was present.

Discussion

In this study we used a next generation sequencing-based transcriptome analysis to characterize mammary gland (MG) tumors from the $p53^{R270H/+}$WAPCre mouse model that were chronically exposed to insulin like molecules. We found indications that tumors of mice that received chronic treatment of X10 or IGF1 have an increased growth potency, enhanced migratory capabilities and a higher Warburg potential. Moreover, the candidate cancer driver mutations in Ezh2 and Hras were highly enriched in X10 and IGF1 tumors.

This is the first study in which a humanized breast cancer mouse model was used to study the tumorigenic effects of chronic insulin analogue treatment. Eventually, all mice from this model developed spontaneous MG tumors with a high human relevance. In this way it was possible to compare tumors induced by insulin analogue treatment with tumors induced by chronic insulin or vehicle treatment. This in contrast to other studies using wild type mice where only a few tumors with an origin less relevant to the human situation could be evaluated [174] [175].

Tumors often have an increased IRA:IRB ratio [206] [135] that can possibly influence the in vivo transformational effects of insulin analogues [207]. We found that IRA gene expression levels are up regulated (2 fold) in pre-neoplastic MGs compared to expression levels in normal MGs, but IRA gene expression levels in tumors were similar to that of normal MG tissue. This indicates that the IRA might play a role in the transformation of normal to neoplastic MG tissue, but once the MG tumor is established the A isoform of the insulin receptor does not play a key role in proliferative signalling anymore. Surprisingly, IRB gene expression levels were strongly down regulated (over 10 fold) in MG tumors. This suggests that the IRA:IRB ratio is indeed increased in MG tumors, but this effect is mainly caused by down regulation of IRB expression levels rather than an up regulation of IRA.

We anticipated that chronic stimulation with insulin-like molecules would decrease the Warburg potency, as insulin deprivation in human fibroblast led to an induction of anaerobic glycolysis [208]. Indeed, we saw that 90% of the insulin induced tumors showed an increased oxidative phosphorylation response compared to 60% for the spontaneous vehicle induced tumors. Interestingly, we found that compounds that induced an equi-glycaemic response but with an increasing mitogenic potential at the doses used in this study (insulin, glargine, X10 and IGF1) [194] also showed an increasing percentage of tumors depending on anaerobic glycolysis (10%, 30%, 50% and 60% resp.). This might suggest that proliferative signalling is indirectly or directly coupled to the Warburg effect. Tumor samples were the samples have deliberately been taken 24 hours after the last injection, since we were interested in the long term rather than the short term in vivo effects. During this time the exogenous compounds are fully degraded by enzymes and

therefore no short-term signalling effect of the compounds can be observed. [131] [83]. However, we cannot fully exclude the effect of direct insulin analogue treatment on tumor cell metabolism, since also treatment of MCF7-IGF1R cells to the various insulin analogues did affect the glycolytic metabolic program.

Using the SVM model we could detect a sustained proliferative signalling in the chronic X10/IGF1 treated tumors, which might suggest that chronic growth factor treatment can transform tumors in such a way that an autocrine growth factor signalling pathway is induced. A likely explanation would be differential mutational pattern in cancer driver genes that underlie such a differential proliferative pathway. Chronic insulin, glargine, X10 or IGF1 treatment did not result in more mutations and no correlation could be detected with number of mutations and tumor latency time. Interestingly several X10/IGF1 enriched mutations were observed, including Ezh2, Hras and Traf7, of which Ezh2 and Hras are prominent modulators of human breast cancer. Possibly these specific mutations contribute (in)-directly to the X10/IGF1 phenotypes and enhanced tumor development and progression.

Altogether our data suggest that the observed decreased tumor latency time in the $p53^{R270H/+}$WAPCre mouse model after chronic X10/IGF1 treatment is a result of an enhanced tumor biomass production rate. Furthermore these treatments might facilitate tissue invasion and metastasis and deregulate the cellular metabolism in the tumor. All these factors contribute to an enhanced tumor development and thus decreasing the MG tumor latency time in this model. We did not find evidence that chronic glargine treatment induced a more aggressive tumor phenotype or increased the biomass production rate, but a slight increased Warburg potential was observed compared to tumors induced by insulin treatment.

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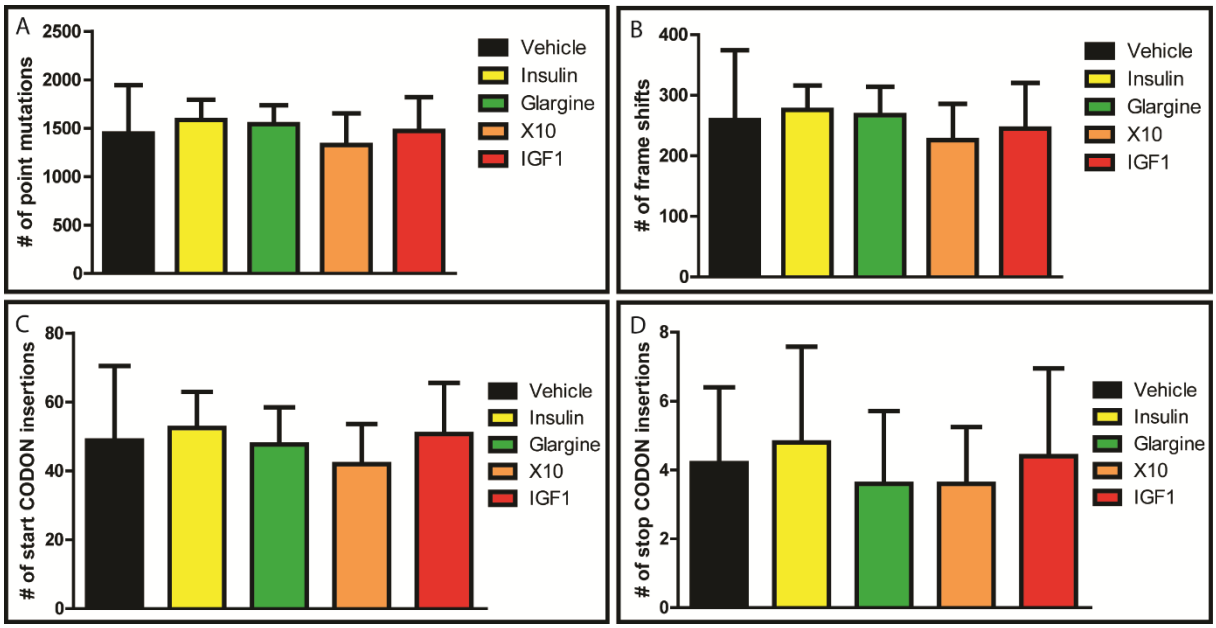
Duality of interest

There is no duality of interest that could be perceived as prejudicing the impartiality of the research reported. None of the authors declares any conflict of interest.

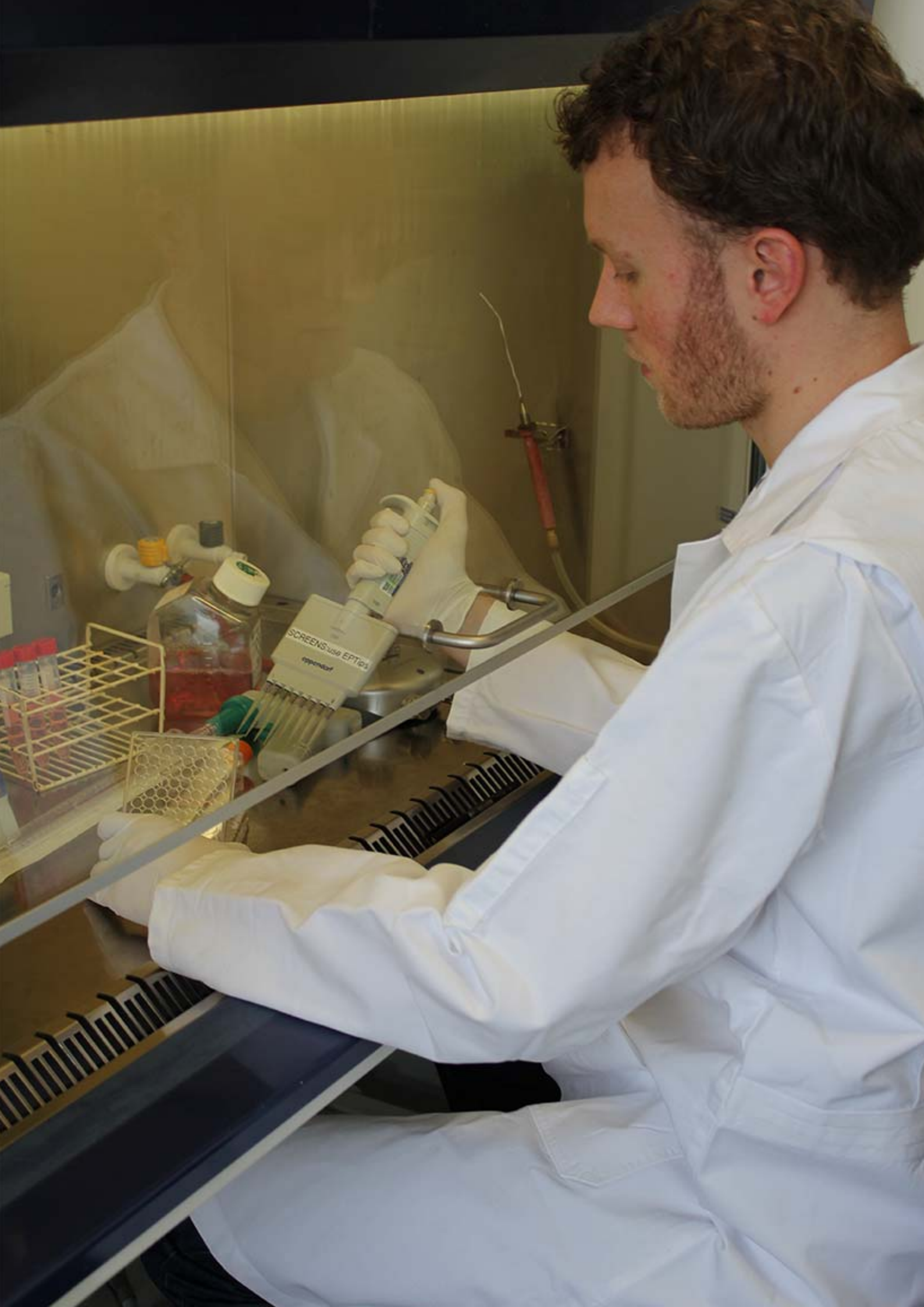
Contribution statement

Conceived and designed the experiments: BTB, CLES, JL, PJ, ER, BVDW, JWVDL. Performed the experiments: BTB, CV. Gene expression and mutational analysis was performed by PR. SVM and IMAT similations were done by JL. All other data was analyzed by BTB. Wrote the paper: BTB. Reviewed the paper: PR, CLES, CV, ER, BVDW, JWVDL. All authors approved the final version of this paper.

Supplementals



Supplemental figure 1. Chronic insulin analogue treatment does not affect the mutational profile of the tumors. A) The average number of point mutations per treatment group. B) The Average number of frame shifts per treatment. C) The average number of start CODON insertions per treatment and in D) the average number of stop CODON insertions are presented per treatment.



Chapter 7 summary and discussion

Carcinogenicity of insulin analogues: current status and future perspectives

Highlights

- The insulin analogue carcinogenic risk assesment guidelines of the European medicine administration was used as a guidance in our research strategy
- There is no compelling evidence that any commercial available insulin analogue is inducing the risk of cancer
- Glargine has an enhanced mitogenic potential compared to regular insulin and might affect some hallmarks of cancer
- Future research on this topic should include chronic exposure in vivo experiments on humanized genetically engineered mouse models to increase the clinical relevance of these studies

◀ IN THE PICTURE

Cell culture laboratory. In vitro models depend on the culturing of living cells. To prevent the cell culture from being infected with bacteria, fungi or viruses, handling of these cells is performed in special cabinets that create a more or less sterile environment. In these cabinets cells are seeded or harvested, exposures are performed and transfections are executed.

◀ IN BEELD

Cel kweek laboratorium. In vitro modellen berusten op het kweken van levende cellen. Om infecties met bacteriën, schimmels en virussen te voorkomen, zijn de handelingen met deze cellen in speciale cabines gedaan, waarin een (bijna) steriele omgeving is gecreëerd. In deze cabines worden de cellen uitgezaaid, geoogst en worden de blootstellingen en transfecties uitgevoerd.

Biopharmaceutical drugs are medicinal products that have been manufactured or directly extracted from a biological source [209]. These compounds are used for therapeutic purposes, and should therefore be carefully assessed for potential side effects. Several regulatory national and international authorities, like the American FDA or European EMA, are responsible for legislation regarding the safety evaluation of biopharmaceuticals [1, 2].

Growth factors are an example of biopharmaceuticals. They show intrinsic mitogenic behavior and are therefore a problematic group of pharmaceuticals with regard to the carcinogenic safety evaluation, and their inherent proliferative action is a cause for concern [210]. Regulatory agencies have requested the use of specific testing strategies to ensure the safety of a sub-group of growth factors, i.e. insulin analogues [6]. This includes the use of well characterized and validated cell models, state of the art techniques to assess the mitogenic effects and the use of humanized in vivo models. In this thesis we have focused on carcinogenic risk assessment of a specific group of growth factors, the insulin analogues.

With worldwide over 380 million cases, diabetes mellitus is the most common endocrine related disease [142]. Usually, patients with type 1 diabetes depend on daily insulin (analogue) administration to control their blood glucose levels. Small alterations to the protein structure of insulin affect the pharmacokinetics and dynamics of the molecule, and consequently cause these insulin-like molecules to either act faster (rapid-acting analogues) or slower (long-acting analogues) than regular insulin. Often a combination treatment of these long- and short-acting insulin analogues is used to mimic the endogenous insulin levels in a healthy person throughout the day.

Insulin glargine (LANTUS) is the world's most commonly used long-acting insulin analogue with about 7 million users in 2011 [196]. Three amino acid replacements in the molecular structure of glargine ensure the slow release into the patient's blood and the prolonged action profile [173]. But these mutations have also altered the binding kinetics towards different receptors including the two isoforms of the insulin receptor (IRA and IRB) and the insulin-like growth factor 1 receptor (IGF1R). While activation of the IRB induces the intended metabolic effects (blood glucose drop), it is thought that activation of IRA and IGF1R will contribute to an enhanced mitogenic signaling [211].

Several epidemiological studies have suggested that the use of some of these insulin analogues is correlated with an increased incidence of cancer, especially breast cancer [76] [144] [212]. While others could not confirm these findings [19] [146] [71]. Due to the many confounding factors, the interpretation of this type of observational patient studies is often difficult. For example, diabetes mellitus is in itself thought to be a risk factor for cancer [213]. Moreover, insulin analogue dose, treatment duration, and age/gender/lifestyle of patients is often not known/taken into account while these factors are thought to have a strong effect on cancer risk [24]. A patient based random controlled clinical trial setup would overcome these problems, but

so far this type of study on the relationship between insulin analogue treatment versus cancer have been rare and highly underpowered.

It is also possible to study the direct mitogenic and anti-apoptotic effects of these insulin analogues using cell lines. Currently, over fifty in vitro studies are described in literature and sixteen of these studies have specifically focused on breast cancer, using breast (cancer) cell lines. The conclusions drawn from these studies are not always in agreement and sometimes even contradictory [118] [40] [193]. Differences in cell model and experimental procedures are in all likelihood the main cause for these conflicting observations.

If in vitro data are considered insufficient and a cause for concern remains, the evaluation of the carcinogenic risk using an in vivo animal model provides added value. The few chronic exposure experiments with rats and mice that have been described have revealed an increased carcinogenic potential of insulin AspB10 [175], an insulin analogue that therefore never entered the market. All other evaluated insulin analogues, including insulin glargine, did not induce more tumors than regular human insulin.

It is unlikely that growth factors at physiological levels induce tumors, however, at pharmacological levels these compounds might be involved in tumor progression of pre-neoplastic lesions. The disadvantage of using the standard wild type rodent models is the rather low incidence of clinically relevant background tumors. Therefore, the observed carcinogenic effect of the growth factors is very mild in these models. The use of xenograft transplantation in vivo models would overcome this problem [207], but in these models only tumor progression features can be studied and no information can be retrieved about the earlier stages of tumor development.

In this chapter we will describe our view on the testing strategy of insulin analogues in terms of increased mitogenic/carcinogenic potential, bearing in mind the current guidelines on this topic, and we will provide an integrated summary of the results obtained with the models that we used throughout this thesis.

This thesis emphasizes the importance of a proper evaluation of carcinogenic side effects of insulin analogues used by diabetic patients. To ensure the design of a relevant experiment it is of course essential to evaluate the current literature about insulin analogues and cancer risk. Some review studies have summarized part of the available literature on this topic, but so far no systematic review including all in vitro, in vivo (animal) and epidemiological (human) studies is available. In **chapter 2**, we present a systematic review of current literature. We focused on studies that included breast (cancer) cell lines or evaluated the incidence of breast tumors in mice, rats or humans that have been exposed to insulin analogues. The results from the sixteen in vitro, eight animal and thirty epidemiological studies we included were highly diverse. Some studies concluded that insulin analogues, especially glargine, did have carcinogenic/mitogenic effects, while others found no effect or even milder carcinogenic/mitogenic effects compared to

regular insulin. All in all we found no compelling evidence that any clinically available insulin analogue increases breast cancer risk. However, both in vitro and epidemiological studies suffered from methodological limitations. Besides, epidemiological studies were underpowered and animal data was scarce. Therefore, there is a strong need for well-designed studies that evaluate the carcinogenic and mitogenic potential of insulin analogues.

A comprehensive evaluation should start with in vitro studies, performed in well characterized cell lines. Since the IGF1R is likely to be the main receptor regulating the mitogenic signaling of insulin analogues, it is crucial that the used cell line expresses this receptor, and at high levels if possible. Furthermore, the concentration at which the analogues are tested is very important. Using concentrations that are too low might lead to undetectable signaling events whereas the use of overtly high concentrations will overstimulate the receptor. In both cases one is unable to discriminate between the mitogenic effects of the different compounds. Thus, a broad concentration range is preferred. In addition, the use of positive and negative controls is essential. Without including regular insulin and IGF1 as a reference compounds, it is difficult to put the obtained results in perspective and provide a quantitative mitogenic score for individual analogues. **Chapter 3** describes an in vitro study in which we tested all commercially available insulin analogues using a human breast cancer cell line panel that expresses either one of the isoforms of the insulin receptor (IRA/IRB) or the IGF1R at high levels. The activation of downstream signaling was evaluated upon insulin analogue treatment. We found that treatment with insulin glargine or AspB10 induced a significantly stronger activation of the MAPK and the PI3K signaling pathways, compared to regular insulin, especially via the IGF1R. With a functional assay we tested the direct proliferative effects of these compounds using a concentration range from physiological to supra-physiological levels. We found that AspB10, though not glargine, had a significant higher proliferative potential compared to regular insulin. We demonstrated that the glargine was rapidly degraded, into metabolites with a low mitogenic potency, by enzymes present in the plasma. This conversion could also explain the difference we observed between the results of the SRB proliferation assay (in which serum was present and thus the glargine was converted) and the WB experiments (in which no serum was added and most of the glargine was still present). Since this bioconversion of glargine is also relevant for the human in vivo situation we tested the mitogenic potential of the two main metabolites of glargine and found that both metabolites (M1 and M2) have a low mitogenic potential similar to that of regular insulin. In conclusion, glargine had a strong intrinsic mitogenic potential but due to the rapid degradation into metabolites with a low proliferative behavior there was no sustained activation of the IGF1R signaling pathway. All other tested commercially available insulin analogues showed a mitogenic potential that was comparable to regular insulin.

In **chapter 4,** we performed a stimulation experiment using the same cell line panel as described in chapter 3. This time mRNA was extracted and a full transcriptomic analysis was performed

using Affymetrix micro-arrays. This allowed us to study the insulin analogue induced mitogenic signaling cascades in more detail. In general, activation of the IRA resulted in a transcriptomic response similar to that of IGF1R. Only AspB10 stimulation resulted in a very distinct gene expression profile via the IRA, which is in line with an older study that showed a prolonged IRA occupancy time for AspB10, therefore it was suggested that AspB10 induces its mitogenic signaling especially via the IRA [149]. We identified a set of genes that was significantly upregulated upon IGF1 and AspB10 stimulation but to a much milder extent after regular insulin stimulation, suggesting that these genes could act as transcriptomic markers for mitogenic signaling. Examples of these markers are the early growth response (EGR) genes, all four EGR genes were picked up by our assay. Although these genes are well known to play a role in proliferation, so far they have not been linked to insulin receptor (ISNR)/IGF1R or insulin analogue signaling. Next we evaluated the expression of these genes after stimulation with other commercially available insulin analogues. The mitogenic score, or absolute expression value of these genes, showed a clear correlation with the mitogenic indexes as calculated from functional bioassays as well as receptor kinetic studies in literature [114] [113]. Furthermore, we were able to confirm the robustness of some of these classifiers in mammary gland tissue of an insulin injected mice as well as insulin analogue stimulated human primary (non-cancer) mammary cells. Although more research is needed to determine the clinical relevance of these mitogenic classifiers, we think that this straightforward RT-Q-PCR based analysis has the potential to become a quick alternative for a mitogenic screen of compounds that act on the IGF1R.

As mentioned before, glargine itself has a strong mitogenic potential, but due to the rapid enzymatic conversion to low mitogenic compounds, this might not be relevant in vivo. Due to uncertainties about the exact rate of conversion and therefore possible exposure to parental and mitogenic glargine, an in vivo experiment would provide further insight into glargine induced carcinogenicity. In **chapter 5** a chronic exposure experiment is described, in which we injected mice every other day with a high dose of insulin, glargine, AspB10, IGF1 or a vehicle solution. For this the $p53^{R270H/+}$WAPCre mouse model was used. In this model, mice will develop spontaneous human relevant mammary gland tumors. We found that frequent AspB10 and IGF1 injections significantly decreased the latency time for breast tumor development, while chronic glargine exposure showed a slight non-significant decrease on tumor latency time. The only other chronic in vivo experiment that included glargine as one of the test compounds did not find any effect in terms of tumor latency time [175]. The time to develop a mammary gland tumor was not affected by regular insulin injections compared to the tumors in the vehicle treatment group. Insulin analogue treatment in general did not affect the tumor type in this mouse model and cell lines isolated from these tumors did not reveal an enhanced aggressiveness or invasiveness. This was in agreement with a study on diabetic breast cancer patients in which clinical and

histopathological screening of the tumors revealed that glargine treatment did not affect the tumor stage compared to regular insulin treatment [93]. However, we found that the protein expression profiles of AspB10 and IGF1 treated tumors showed an enhanced/sustained activation of the PI3K and MAPK signaling cascades, which are thought to be the main driver for tumor development. These pathways were also upregulated in glargine induced tumors. A metabolite analysis revealed that similar to the human situation, the glargine was rapidly converted by enzymes in the blood. Nevertheless, in contrast to the human situation this enzymatic conversion seemed to be somewhat slower in mice as we were still able to detect low concentrations of glargine in the blood one hour after glargine injection [83] [140]. Furthermore, besides very high concentrations of M1 we were able to detect also some M2, which was not observed in the human studies. These observations suggest that glargine dynamics is slightly different from mice to man and highlight the need for studies that examine the differences between these species.

The altered protein expression in the glargine induced tumors together with the slight decrease in tumor latency time suggests that frequent glargine injections might affect tumorigenesis in this mouse model to some degree. To further investigate this, mRNA from fifty mammary gland tumors was isolated and analyzed using next generation sequencing. This whole transcriptome analysis is described in **chapter 6**. This is the first study in which such an experiment was performed on the most clinically relevant target tissue (and not just the standard liver tissue). Several "Hallmarks of Cancer" appeared to be affected by chronic insulin analogue treatment [214] [197]. AspB10 and IGF1 treated tumors showed an increased and sustained proliferative and invasive potential. These tumors as well as the tumors treated with glargine also relied more on aerobic glycolysis than on oxidative phosphorylation for their ATP generation, which is the so-called Warburg effect. To our knowledge, such a link between insulin analogue signaling and the Warburg effect has never been described. Mutational analysis indicated that chronic insulin analogue stimulation did not affect the genetic instability of the tumors, since no additional mutations were found in the treatment groups. However enrichment of *Ezh1* and *Hras* were found in tumors of mice treated with glargine, X10 or IGF1. Furthermore we found that chronic AspB10 and IGF1 treatment resulted in tumors with highly induced metabolic processes that are involved in biomass production. This confirms the hypothesis that chronic AspB10 and IGF1 treatments decrease tumor latency time in the $p53^{R270H/+}$WAPCre mouse model by inducing tumor progression rather than tumor initiation.

The work described in this thesis provides novel insights into the role of insulin analogues in cancer and deepens the recommendations of ''the points to consider document'' of the EMA [6]. Our in vitro studies included pathway activation studies, functional proliferation studies as well as an in depth transcriptomic analysis. This in combination with the use of a well-characterized MCF7-based cell model provided new information regarding the mitogenic mode of action of

the insulin analogues and contributed to the inter-species-translatory aspects of these assays. To further follow the EMA guidelines we were able to validate some of the transcriptomic results in human primary mammary cells. Future in vitro research on this topic should contain other functional read-outs (e.g. MTT or BrdU proliferation assays), as well as FACS-assays to determine a shift in cell cycle upon insulin analogue stimulation. It would be ideal to have these additional tests to be performed in human primary mammary cells as such a model is clinically more relevant and therefore the true carcinogenesis (the development of a cancerous cell) can be better assessed.

In the EMA guidelines concerning carcinogenic risk assessment of insulin analogues the use of in vivo experiments is encouraged. It is thought that in carcinogenicity testing the rat represents the human situation better as a model compared to mice [4]. However, there are hardly any humanized breast cancer rat models available. For this reason we used the $p53^{R270H/+}$WAPCre mouse model. All mice in this model develop spontaneous mammary tumors, so we could test if the insulin analogues affect tumorigenesis either by decreasing the tumor latency time, increasing the tumor multiplicity or affecting tumor type. The main mammary gland tumors that developed in our mouse model were characterized as EMT tumors. These tumors make up only a small fraction of the human tumors. Future chronic in vivo studies should use humanized breast cancer mouse models with e.g. a PI3K mutation predispositioned to develop carcinomas to increase the clinical relevance of their study [215] [190].

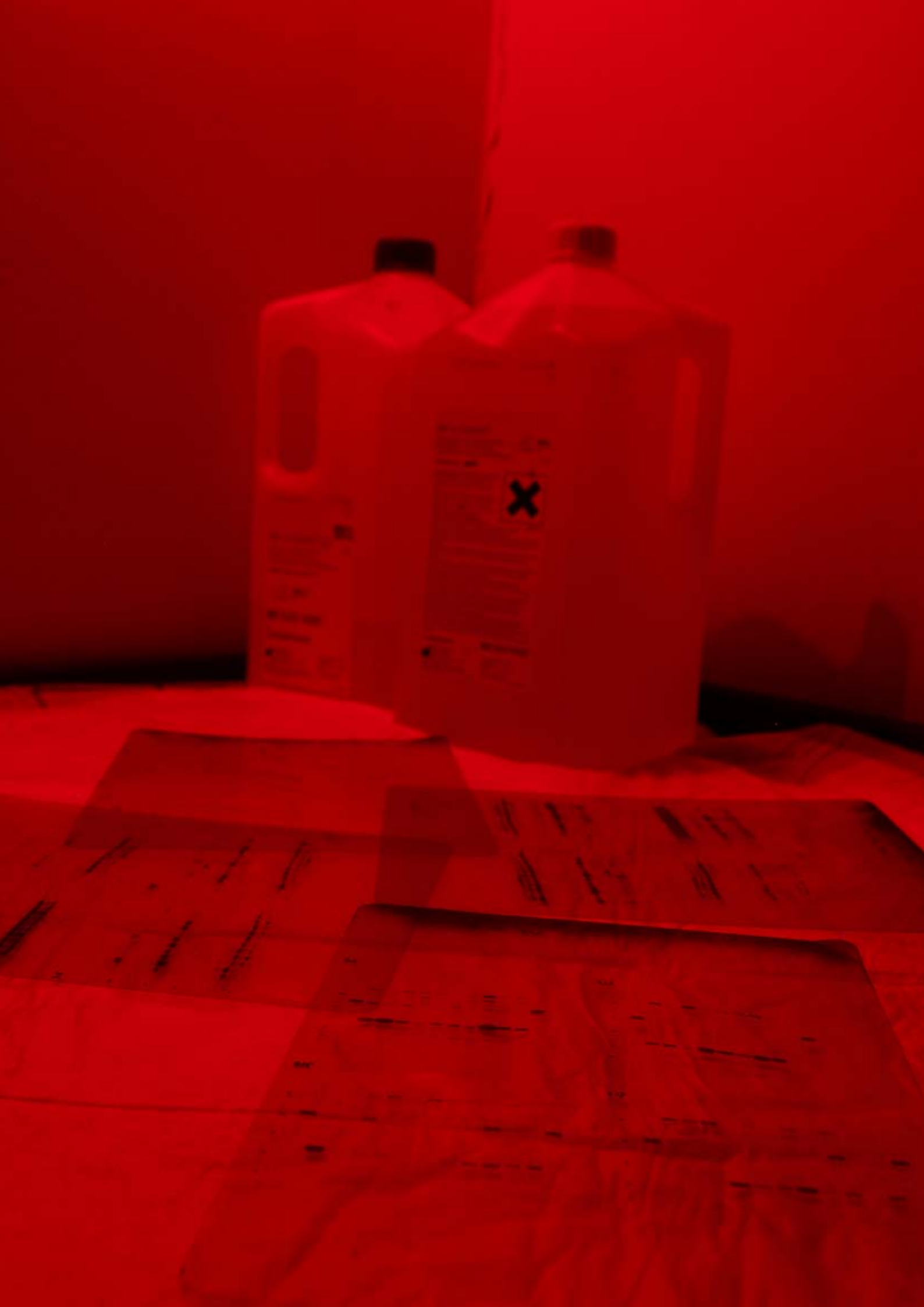
Although more research is needed, the available data suggests that chronic glargine treatment is not involved in tumor initiation but might influence several features of tumorigenesis, and thus it might enhance tumor progression [193]. We suggest that future epidemiological studies (preferably case control studies) should focus on these potentially tumor promoting effects rather than on the tumor initiating effects of glargine. Until this research is performed we advise people with a high risk of cancer (e.g. patients that have already been diagnosed with cancer, survivors of oncogenic diseases or people with a familial genetic predisposition to develop this disease) to reconsider their glargine prescription. Alternative diabetic treatments, like the use of long acting insulin determir or the bioglycolin metformin, are in fact sometimes correlated with a decreased incidence of cancer [216] [58].

For the future it is essential that regulatory agencies react quickly, yet not overhasty to indications from scientific studies that a specific compound might possess an increased carcinogenic risk. In 2009, concerns about the carcinogenic potential of glargine were raised by an epidemiological study by Hemkens and colleagues [16]. Instead of directly publishing this article and thereby risking the possibly of setting of an unwarranted alarm, the European Association for the Study of Diabetes (EASD) asked several other epidemiological research groups to study the same topic on different patient groups. Within a couple of months three other epidemiological studies were performed and published together with the study of

Hemkens [17-19]. The other studies found no correlation or only a correlation with breast cancer and the use of glargine treatment. A response from the European regulatory agencies was slightly delayed, but in 2011 a new FP7 "Cancer risk and insulin analogues" (CARING) program was initiated. This international collaboration aims to quantify the risk of cancer associated with the long-term use of insulin and insulin analogues, with the main focus on epidemiological research. The review in chapter 2 is a direct product of this program. In my opinion, also an additional guideline should have been proposed by these ministries directly after the appearance of the epidemiological studies regarding follow up in vitro and in vivo studies on this topic. Yet, to this day no addendum has been proposed on "The points to consider document" of the EMA. I expect that such a recommendation from these organizations would have led to more and higher quality studies in a shorter timeframe.

In this thesis the recommendations of the ICHS6 guideline and "The points to consider document" of EMA were followed for the carcinogenic risk assessment of all commercial insulin analogues [1, 2, 6]. Although we could propose some improvements for the used models (see suggestions as discussed above), we think that the research described in this thesis provides the most accurate, complete and in-depth carcinogenic safety evaluation for insulin analogues to date. We hope that with this research we have contributed to the improvement of the quality and consistency of the preclinical safety data supporting the development of insulin analogues.

7



References

◀ IN THE PICTURE

Dark room with red light. This room was used to develop the light-sensitive Western blot films (in front of the picture). The exposed film was processed by immersing it in developing solution (container with black lid), fixed by thiosulfates (container with red lid) and cleaned with water. The films are only sensitive to blue/green light, thus red light enables the scientist to see what he is doing without exposing the film. Nowadays there are light sensitive imagers that can detect chemiluminescent light and immediately convert it to a digital image. This process might be quicker, but I prefer the old fashioned way.

◀ IN BEELD

Donkere kamer met rood licht. Deze kamer werd gebruikt om licht gevoelige Western blot films te ontwikkelen. De blootgestelde film werd verwerkt door hem onder te dompelen in ontwikkelingsoplossing (tank met zwarte dop), gefixeerd met tiosulfaten (tank met rode dop) en schoongemaakt met water. De films zijn alleen gevoelig voor blauw/groen licht. Met behulp van het rode licht kan de onderzoeker zien waarmee hij bezig is zonder de film bloot te stellen. Tegenwoordig worden ook wel lichtgevoelige scanners gebruikt voor de detectie van lichtgevoelige films. Deze scanners zijn een stuk sneller.

1. ICHS6(R1): **Preclinical safety evaluation of biotechnology-derived pharmaceuticals**. In.; 1997.
2. ICHS6(R1): **Addendum – preclinical safety evaluation of biotechnology-derived pharmaceuticals** In.; 2011.
3. Vahle JL, Finch GL, Heidel SM, Hovland DN, Jr., Ivens I, Parker S, Ponce RA, Sachs C, Steigerwalt R, Short B *et al*: **Carcinogenicity assessments of biotechnology-derived pharmaceuticals: a review of approved molecules and best practice recommendations**. *Toxicol Pathol* 2010, **38**(4):522-553.
4. Van Oosterhout JP, Van der Laan JW, De Waal EJ, Olejniczak K, Hilgenfeld M, Schmidt V, Bass R: **The utility of two rodent species in carcinogenic risk assessment of pharmaceuticals in Europe**. *Regulatory toxicology and pharmacology : RTP* 1997, **25**(1):6-17.
5. Friedrich A, Olejniczak K: **Evaluation of carcinogenicity studies of medicinal products for human use authorised via the European centralised procedure (1995-2009)**. *Regulatory toxicology and pharmacology : RTP* 2011, **60**(2):225-248.
6. EMA: **Points to consider document on the non-clinical assessment of the carcinogenic potential of insulin analogues,** **http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003252.pdf**. In.; 2002.
7. IARC: **breast cancer fact sheet**. 2012.
8. Peairs Ks Fau - Barone BB, Barone Bb Fau - Snyder CF, Snyder Cf Fau - Yeh H-C, Yeh Hc Fau - Stein KB, Stein Kb Fau - Derr RL, Derr Rl Fau - Brancati FL, Brancati Fl Fau - Wolff AC, Wolff AC: **Diabetes mellitus and breast cancer outcomes: a systematic review and meta-analysis**. (1527-7755 (Electronic)).
9. Larsson Sc Fau - Mantzoros CS, Mantzoros Cs Fau - Wolk A, Wolk A: **Diabetes mellitus and risk of breast cancer: a meta-analysis**. (0020-7136 (Print)).
10. Liao S Fau - Li J, Li J Fau - Wei W, Wei W Fau - Wang L, Wang L Fau - Zhang Y, Zhang Y Fau - Li J, Li J Fau - Wang C, Wang C Fau - Sun S, Sun S: **Association between diabetes mellitus and breast cancer risk: a meta-analysis of the literature**. (1513-7368 (Print)).
11. Xue F Fau - Michels KB, Michels KB: **Diabetes, metabolic syndrome, and breast cancer: a review of the current evidence**. (0002-9165 (Print)).
12. Starup-Linde J, Karlstad O, Eriksen SA, Vestergaard P, Bronsveld HK, de Vries F, Andersen M, Auvinen A, Haukka J, Hjellvik V *et al*: **CARING (CAncer Risk and INsulin analoGues): the association of diabetes mellitus and cancer risk with focus on possible determinants - a systematic review and a meta-analysis**. *Curr Drug Saf* 2013, **8**(5):296-332.
13. De Bruijn KM, Arends Lr Fau - Hansen BE, Hansen Be Fau - Leeflang S, Leeflang S Fau - Ruiter R, Ruiter R Fau - van Eijck CHJ, van Eijck CH: **Systematic review and meta-analysis of the association between diabetes mellitus and incidence and mortality in breast and colorectal cancer**. (1365-2168 (Electronic)).
14. Home P: **Insulin therapy and cancer**. *Diabetes Care* 2013, **36 Suppl 2**:S240-244.
15. Pollak M, Russell-Jones D: **Insulin analogues and cancer risk: cause for concern or cause celebre?** *Int J Clin Pract* 2010, **64**(5):628-636.
16. Hemkens LG, Grouven U, Bender R, Gunster C, Gutschmidt S, Selke GW, Sawicki PT: **Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study**. *Diabetologia* 2009, **52**(9):1732-1744.
17. Jonasson JM, Ljung R, Talback M, Haglund B, Gudbjornsdottir S, Steineck G: **Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden**. *Diabetologia* 2009, **52**(9):1745-1754.
18. Colhoun HM: **Use of insulin glargine and cancer incidence in Scotland: a study from the Scottish Diabetes Research Network Epidemiology Group**. *Diabetologia* 2009, **52**(9):1755-1765.
19. Currie CJ, Poole CD, Gale EA: **The influence of glucose-lowering therapies on cancer risk in type 2 diabetes**. *Diabetologia* 2009, **52**(9):1766-1777.
20. Butler PC: **Insulin glargine controversy: a tribute to the editorial team at Diabetologia**. *Diabetes* 2009, **58**(11):2427-2428.
21. Mollentze WF: **A possible link between insulin glargine and malignancy: the facts**. *Cardiovasc J Afr* 2009, **20**(4):216-218; discussion 218-219.
22. Werner H, Chantelau EA: **Differences in bioactivity between human insulin and insulin analogues approved for therapeutic use- compilation of reports from the past 20 years**. *Diabetology & Metabolic Syndrome* 2011, **3**.

23. Ciaraldi TP, Sasaoka T: **Review on the in vitro interaction of insulin glargine with the insulin/insulin-like growth factor system: potential implications for metabolic and mitogenic activities**. *Horm Metab Res* 2011, **43**(1):1-10.
24. Janghorbani M, Dehghani M, Salehi-Marzijarani M: **Systematic review and meta-analysis of insulin therapy and risk of cancer**. *Horm Cancer* 2012, **3**(4):137-146.
25. Karlstad O Fau - Starup-Linde J, Starup-Linde J Fau - Vestergaard P, Vestergaard P Fau - Hjellvik V, Hjellvik V Fau - Bazelier MT, Bazelier Mt Fau - Schmidt MK, Schmidt Mk Fau - Andersen M, Andersen M Fau - Auvinen A, Auvinen A Fau - Haukka J, Haukka J Fau - Furu K, Furu K Fau - de Vries F *et al*: **Use of insulin and insulin analogs and risk of cancer - systematic review and meta-analysis of observational studies**. (2212-3911 (Electronic)).
26. Colmers IN, Bowker SL, Tjosvold LA, Johnson JA: **Insulin use and cancer risk in patients with type 2 diabetes: a systematic review and meta-analysis of observational studies**. *Diabetes Metab* 2012, **38**(6):485-506.
27. Tang X, Yang L, He Z, Liu J: **Insulin glargine and cancer risk in patients with diabetes: a meta-analysis**. *PLoS One* 2012, **7**(12):e51814.
28. Du X, Zhang R, Xue Y, Li D, Cai J, Zhou S, Huang Z, Yu R, Liu Y: **Insulin glargine and risk of cancer: a meta-analysis**. *The International journal of biological markers* 2012, **27**(3):e241-246.
29. PROSPERO: **http://www.crd.york.ac.uk/PROSPERO/**.
30. Moher D, Liberati A Fau - Tetzlaff J, Tetzlaff J Fau - Altman DG, Altman DG: **Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement**. (1549-1676 (Electronic)).
31. The Cochrane Collaboration. Higgins JPT GS, eds.: **Cochrane Handbook for Systematic Reviews of Interventions, Version 5.1.0 [updated March 2011]**. 2011.
32. Gallagher EJ, Alikhani N, Tobin-Hess A, Blank J, Buffin NJ, Zelenko Z, Tennagels N, Werner U, LeRoith D: **Insulin receptor phosphorylation by endogenous insulin or the insulin analog AspB10 promotes mammary tumor growth independent of the IGF-I receptor**. *Diabetes* 2013, **62**(10):3553-3560.
33. Glendorf T, Knudsen L, Stidsen CE, Hansen BF, Hegelund AC, Sorensen AR, Nishimura E, Kjeldsen T: **Systematic evaluation of the metabolic to mitogenic potency ratio for B10-substituted insulin analogues**. *PLoS One* 2012, **7**(2):e29198.
34. Hansen BF, Glendorf T, Hegelund AC, Lundby A, Lutzen A, Slaaby R, Stidsen CE: **Molecular characterisation of long-acting insulin analogues in comparison with human insulin, IGF-1 and insulin X10**. *PLoS One* 2012, **7**(5):e34274.
35. Knudsen L, Hansen BF, Jensen P, Pedersen TA, Vestergaard K, Schaffer L, Blagoev B, Oleksiewicz MB, Kiselyov VV, De Meyts P: **Agonism and antagonism at the insulin receptor**. *PLoS One* 2012, **7**(12):e51972.
36. Liefvendahl E, Arnqvist HJ: **Mitogenic effect of the insulin analogue glargine in malignant cells in comparison with insulin and IGF-I**. *Hormone and Metabolic Research* 2008, **40**(6):369-374.
37. Mayer D, Shukla A, Enzmann H: **Proliferative effects of insulin analogues on mammary epithelial cells**. *Arch Physiol Biochem* 2008, **114**(1):38-44.
38. Milazzo G, Sciacca L, Papa V, Goldfine ID, Vigneri R: **ASPB10 insulin induction of increased mitogenic responses and phenotypic changes in human breast epithelial cells: evidence for enhanced interactions with the insulin-like growth factor-I receptor**. *Mol Carcinog* 1997, **18**(1):19-25.
39. Oleksiewicz MB, Bonnesen C, Hegelund AC, Lundby A, Holm GM, Jensen MB, Krabbe JS: **Comparison of intracellular signalling by insulin and the hypermitogenic AspB10 analogue in MCF-7 breast adenocarcinoma cells**. *J Appl Toxicol* 2011, **31**(4):329-341.
40. Pierre-Eugene C, Pagesy P, Nguyen TT, Neuille M, Tschank G, Tennagels N, Hampe C, Issad T: **Effect of Insulin Analogues on Insulin/IGF1 Hybrid Receptors: Increased Activation by Glargine but Not by Its Metabolites M1 and M2**. *PLoS One* 2012, **7**(7):e41992.
41. Sciacca L, Cassarino MF, Genua M, Vigneri P, Giovanna Pennisi M, Malandrino P, Squatrito S, Pezzino V, Vigneri R: **Biological Effects of Insulin and Its Analogs on Cancer Cells With Different Insulin Family Receptor Expression**. *J Cell Physiol* 2014.
42. Shukla A, Enzmann H, Mayer D: **Proliferative effect of Apidra (insulin glulisine), a rapid-acting insulin analogue on mammary epithelial cells**. *Arch Physiol Biochem* 2009, **115**(3):119-126.
43. Shukla A, Grisouard J, Ehemann V, Hermani A, Enzmann H, Mayer D: **Analysis of signaling pathways related to cell proliferation stimulated by insulin analogs in human mammary epithelial cell lines**. *Endocr Relat Cancer* 2009, **16**(2):429-441.
44. Staiger K, Hennige AM, Staiger H, Haering HU, Kellerer M: **Comparison of the mitogenic potency of regular human insulin and its analogue glargine in normal and transformed human breast epithelial cells**. *Hormone and Metabolic Research* 2007, **39**(1):65-67.

45. Teng JA, Hou RL, Li DL, Yang RP, Qin J: **Glargine promotes proliferation of breast adenocarcinoma cell line MCF-7 via AKT activation**. *Horm Metab Res* 2011, **43**(8):519-523.
46. Ter Braak B, Siezen CL, Kannegieter N, Koedoot E, van de Water B, van der Laan JW: **Classifying the adverse mitogenic mode of action of insulin analogues using a novel mechanism-based genetically engineered human breast cancer cell panel**. *Arch Toxicol* 2014.
47. Weinstein D, Simon M, Yehezkel E, Laron Z, Werner H: **Insulin analogues display IGF-I-like mitogenic and anti-apoptotic activities in cultured cancer cells**. *Diabetes/metabolism research and reviews* 2009, **25**(1):41-49.
48. Gallagher EJ, Alikhani N, Tobin-Hess A, Cannata D, Tennagels N, Werner U, LeRoith D: **The effect of rhIGF-1 and insulin analogue AspB10 on mammary tumour growth and progression in a mouse model of type 2 diabetes**. *Diabetologia* 2012, **55**:S289.
49. Stammberger I, Bube A, Durchfeld-Meyer B, Donaubauer H, Troschau G: **Evaluation of the carcinogenic potential of insulin glargine (LANTUS) in rats and mice**. *International Journal of Toxicology* 2002, **21**(3):171-179.
50. Stammberger I, Essermeant L: **Insulin glargine: A reevaluation of rodent carcinogenicity findings**. *International Journal of Toxicology* 2012, **31**(2):137-142.
51. Tennagels N, Welte S, Hofmann M, Brenk P, Schmidt R, Werner U: **Differences in metabolic and mitogenic signalling of insulin glargine and insulin aspart B10 in rats**. *Diabetologia* 2013, **56**(8):1826-1834.
52. ter Braak B, Siezen CL, Speksnijder E, Koedoot E, van Steeg H, Salvatori DCF, van de Water B, van der Laan JW: **Mammary gland tumor promotion by chronic administration of IGF1 and the insulin analogue AspB10 in the p53R270H/+WAPCre mouse model**. *Breast Cancer Research (accepted)* 2015.
53. Bodmer M, Meier C, Krahenbuhl S, Jick SS, Meier CR: **Long-term metformin use is associated with decreased risk of breast cancer**. *Diabetes Care* 2010, **33**(6):1304-1308.
54. Bordeleau L, Yakubovich N, Dagenais GR, Rosenstock J, Probstfield J, Yu PC, Ryden LE, Pirags V, Spinas GA, Birkeland KI *et al*: **The Association of Basal Insulin Glargine and/or n-3 Fatty Acids With Incident Cancers in Patients With Dysglycemia**. *Diabetes Care* 2014, **37**(5):1360-1366.
55. Carstensen B, Witte DR, Friis S: **Cancer occurrence in Danish diabetic patients: duration and insulin effects**. *Diabetologia* 2012, **55**(4):948-958.
56. Chang CH, Toh S, Lin JW, Chen ST, Kuo CW, Chuang LM, Lai MS: **Cancer risk associated with insulin Glargine among adult type 2 diabetes patients - a nationwide Cohort study**. *PLoS ONE* 2011, **6**(6).
57. Cleveland RJ, North KE, Stevens J, Teitelbaum SL, Neugut AI, Gammon MD: **The association of diabetes with breast cancer incidence and mortality in the Long Island Breast Cancer Study Project**. *Cancer Causes Control* 2012, **23**(7):1193-1203.
58. Dejgaard A, Lynggaard H, Rastam J, Krogsgaard Thomsen M: **No evidence of increased risk of malignancies in patients with diabetes treated with insulin detemir: a meta-analysis**. *Diabetologia* 2009, **52**(12):2507-2512.
59. Fagot JP, Blotiere PO, Ricordeau P, Weill A, Alla F, Allemand H: **Does insulin glargine increase the risk of cancer compared with other basal insulins? A French nationwide cohort study based on national administrative databases**. *Diabetes Care* 2013, **36**(2):294-301.
60. Ferrara A, Lewis JD, Quesenberry CP, Jr., Peng T, Strom BL, Van Den Eeden SK, Ehrlich SF, Habel LA: **Cohort study of pioglitazone and cancer incidence in patients with diabetes**. *Diabetes Care* 2011, **34**(4):923-929.
61. Grimaldi-Bensouda L, Cameron D Fau - Marty M, Marty M Fau - Barnett AH, Barnett Ah Fau - Penault-Llorca F, Penault-Llorca F Fau - Pollak M, Pollak M Fau - Charbonnel B, Charbonnel B Fau - Riddle M, Riddle M Fau - Mignot L, Mignot L Fau - Boivin J-F, Boivin Jf Fau - Khachatryan A *et al*: **Risk of breast cancer by individual insulin use: an international multicenter study**. (1935-5548 (Electronic)).
62. Gu YJ, Wang CF, Zheng Y, Hou XH, Mo YF, Yu WH, Zhang L, Hu C, Nan HR, Chen L *et al*: **Cancer Incidence and Mortality in Patients with Type 2 Diabetes Treated with Human Insulin: A Cohort Study in Shanghai**. *Plos One* 2013, **8**(1).
63. Habel LA, Danforth KN, Quesenberry CP, Capra A, Van Den Eeden SK, Weiss NS, Ferrara A: **Cohort study of insulin glargine and risk of breast, prostate, and colorectal cancer among patientswith diabetes**. *Diabetes Care* 2013, **36**(12):3953-3960.
64. Home PD, Lagarenne P: **Combined randomised controlled trial experience of malignancies in studies using insulin glargine**. *Diabetologia* 2009, **52**(12):2499-2506.
65. Hsieh MC, Lee TC, Cheng SM, Tu ST, Yen MH, Tseng CH: **The influence of type 2 diabetes and glucose-lowering therapies on cancer risk in the Taiwanese**. *Exp Diabetes Res* 2012, **2012**:413782.
66. Koro C, Barrett S, Qizilbash N: **Cancer risks in thiazolidinedione users compared to other anti-diabetic agents**. *Pharmacoepidemiol Drug Saf* 2007, **16**(5):485-492.

67. Kostev K: **Risk of breast cancer in patients on long-acting insulin analogues in comparison with those on human insulin**. *Diabetologia* 2012, **55**(5):1554-1555.
68. Lind M, Fahlen M, Eliasson B, Oden A: **The relationship between the exposure time of insulin glargine and risk of breast and prostate cancer: An observational study of the time-dependent effects of antidiabetic treatments in patients with diabetes**. *Primary Care Diabetes* 2012, **6**(1):53-59.
69. Ljung R, Talback M Fau - Haglund B, Haglund B Fau - Jonasson JM, Jonasson Jm Fau - Gudbjornsdottir S, Gudbjornsdottir S Fau - Steineck G, Steineck G: **Insulin glargine use and short-term incidence of malignancies - a three-year population-based observation**. 2011(1651-226X (Electronic)).
70. Mannucci E, Monami M, Balzi D, Cresci B, Pala L, Melani C, Lamanna C, Bracali I, Bigiarini M, Barchielli A *et al*: **Doses of insulin and its analogues and cancer occurrence in insulin-treated type 2 diabetic patients**. *Diabetes Care* 2010, **33**(9):1997-2003.
71. Morden NE, Liu SK, Smith J, Mackenzie TA, Skinner J, Korc M: **Further exploration of the relationship between insulin glargine and incident cancer: a retrospective cohort study of older Medicare patients**. *Diabetes Care* 2011, **34**(9):1965-1971.
72. Neumann A, Weill A, Ricordeau P, Fagot JP, Alla F, Allemand H: **Pioglitazone and risk of bladder cancer among diabetic patients in France: a population-based cohort study**. *Diabetologia* 2012, **55**(7):1953-1962.
73. Onitilo AA, Stankowski RV, Berg RL, Engel JM, Glurich I, Williams GM, Doi SA: **Type 2 diabetes mellitus, glycemic control, and cancer risk**. *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP)* 2014, **23**(2):134-140.
74. Redaniel MT, Jeffreys M, May MT, Ben-Shlomo Y, Martin RM: **Associations of type 2 diabetes and diabetes treatment with breast cancer risk and mortality: a population-based cohort study among British women**. *Cancer Causes Control* 2012, **23**(11):1785-1795.
75. Rosenstock J, Fonseca V, McGill JB, Riddle M, Halle JP, Hramiak I, Johnston P, Davis M: **Similar risk of malignancy with insulin glargine and neutral protamine Hagedorn (NPH) insulin in patients with type 2 diabetes: findings from a 5 year randomised, open-label study**. *Diabetologia* 2009, **52**(9):1971-1973.
76. Ruiter R, Visser LE, van Herk-Sukel MP, Coebergh JW, Haak HR, Geelhoed-Duijvestijn PH, Straus SM, Herings RM, Stricker BH: **Risk of cancer in patients on insulin glargine and other insulin analogues in comparison with those on human insulin: results from a large population-based follow-up study**. *Diabetologia* 2012, **55**(1):51-62.
77. Sturmer T, Marquis MA, Zhou H, Meigs JB, Lim S, Blonde L, Macdonald E, Wang R, Lavange LM, Pate V *et al*: **Cancer incidence among those initiating insulin therapy with glargine versus human NPH insulin**. *Diabetes Care* 2013, **36**(11):3517-3525.
78. Suissa S, Azoulay L, Dell'Aniello S, Evans M, Vora J, Pollak M: **Long-term effects of insulin glargine on the risk of breast cancer**. *Diabetologia* 2011, **54**(9):2254-2262.
79. Vallarino C, Perez A, Fusco G, Liang H, Bron M, Manne S, Joseph G, Yu S: **Comparing pioglitazone to insulin with respect to cancer, cardiovascular and bone fracture endpoints, using propensity score weights**. *Clinical drug investigation* 2013, **33**(9):621-631.
80. Jorgensen L, Dideriksen L, Drejer K: **CARCINOGENIC EFFECT OF THE HUMAN INSULIN ANALOG B10 ASP IN FEMALE RATS**. In: *Diabetologia: 1992*: SPRINGER VERLAG 175 FIFTH AVE, NEW YORK, NY 10010; 1992: A3-A3.
81. Sommerfeld MR, Muller G Fau - Tschank G, Tschank G Fau - Seipke G, Seipke G Fau - Habermann P, Habermann P Fau - Kurrle R, Kurrle R Fau - Tennagels N, Tennagels N: **In vitro metabolic and mitogenic signaling of insulin glargine and its metabolites**. (1932-6203 (Electronic)).
82. Kuerzel GU, Shukla U Fau - Scholtz HE, Scholtz He Fau - Pretorius SG, Pretorius Sg Fau - Wessels DH, Wessels Dh Fau - Venter C, Venter C Fau - Potgieter MA, Potgieter Ma Fau - Lang AM, Lang Am Fau - Koose T, Koose T Fau - Bernhardt E, Bernhardt E: **Biotransformation of insulin glargine after subcutaneous injection in healthy subjects**. (0300-7995 (Print)).
83. Bolli GB, Hahn AD, Schmidt R, Eisenblaetter T, Dahmen R, Heise T, Becker RH: **Plasma exposure to insulin glargine and its metabolites m1 and m2 after subcutaneous injection of therapeutic and supratherapeutic doses of glargine in subjects with type 1 diabetes**. *Diabetes Care* 2012, **35**(12):2626-2630.
84. Werner H, Chantelau EA: **Differences in bioactivity between human insulin and insulin analogues approved for therapeutic use- compilation of reports from the past 20 years**. (1758-5996 (Electronic)).
85. Van Oosterhout JP, Van der Laan Jw Fau - De Waal EJ, De Waal Ej Fau - Olejniczak K, Olejniczak K Fau - Hilgenfeld M, Hilgenfeld M Fau - Schmidt V, Schmidt V Fau - Bass R, Bass R: **The utility of two rodent species in carcinogenic risk assessment of pharmaceuticals in Europe**. (0273-2300 (Print)).

86. Gerstein HC, Bosch J, Dagenais GR, Diaz R, Jung H, Maggioni AP, Pogue J, Probstfield J, Ramachandran A, Riddle MC *et al*: **Basal insulin and cardiovascular and other outcomes in dysglycemia**. *N Engl J Med* 2012, **367**(4):319-328.
87. Fleming ST, Rastogi A Fau - Dmitrienko A, Dmitrienko A Fau - Johnson KD, Johnson KD: **A comprehensive prognostic index to predict survival based on multiple comorbidities: a focus on breast cancer**. (0025-7079 (Print)).
88. Hou G, Zhang S Fau - Zhang X, Zhang X Fau - Wang P, Wang P Fau - Hao X, Hao X Fau - Zhang J, Zhang J: **Clinical pathological characteristics and prognostic analysis of 1,013 breast cancer patients with diabetes**. (1573-7217 (Electronic)).
89. Liao S, Li J Fau - Wang L, Wang L Fau - Zhang Y, Zhang Y Fau - Wang C, Wang C Fau - Hu M, Hu M Fau - Ma B, Ma B Fau - Wang G, Wang G Fau - Sun S, Sun S: **Type 2 diabetes mellitus and characteristics of breast cancer in China**. (1513-7368 (Print)).
90. Luo J, Virnig B Fau - Hendryx M, Hendryx M Fau - Wen S, Wen S Fau - Chelebowski R, Chelebowski R Fau - Chen C, Chen C Fau - Rohan T, Rohan T Fau - Tinker L, Tinker L Fau - Wactawski-Wende J, Wactawski-Wende J Fau - Lessin L, Lessin L Fau - Margolis K *et al*: **Diabetes, diabetes treatment and breast cancer prognosis**. (1573-7217 (Electronic)).
91. Srokowski TP, Fang S Fau - Hortobagyi GN, Hortobagyi Gn Fau - Giordano SH, Giordano SH: **Impact of diabetes mellitus on complications and outcomes of adjuvant chemotherapy in older patients with breast cancer**. (1527-7755 (Electronic)).
92. van de Poll-Franse LV, Houterman S Fau - Janssen-Heijnen MLG, Janssen-Heijnen Ml Fau - Dercksen MW, Dercksen Mw Fau - Coebergh JWW, Coebergh Jw Fau - Haak HR, Haak HR: **Less aggressive treatment and worse overall survival in cancer patients with diabetes: a large population based analysis**. (0020-7136 (Print)).
93. Besic N, Satej N: **Insulin glargine versus other types of basal insulin-clinical and tumor characteristics in patients with breast carcinoma**. *BMC Res Notes* 2013, **6**:416.
94. Cleveland RJ, North Ke Fau - Stevens J, Stevens J Fau - Teitelbaum SL, Teitelbaum Sl Fau - Neugut AI, Neugut Ai Fau - Gammon MD, Gammon MD: **The association of diabetes with breast cancer incidence and mortality in the Long Island Breast Cancer Study Project**. (1573-7225 (Electronic)).
95. Currie CJ, Poole Cd Fau - Jenkins-Jones S, Jenkins-Jones S Fau - Gale EAM, Gale Ea Fau - Johnson JA, Johnson Ja Fau - Morgan CL, Morgan CL: **Mortality after incident cancer in people with and without type 2 diabetes: impact of metformin on survival**. (1935-5548 (Electronic)).
96. Erickson K, Patterson Re Fau - Flatt SW, Flatt Sw Fau - Natarajan L, Natarajan L Fau - Parker BA, Parker Ba Fau - Heath DD, Heath Dd Fau - Laughlin GA, Laughlin Ga Fau - Saquib N, Saquib N Fau - Rock CL, Rock Cl Fau - Pierce JP, Pierce JP: **Clinically defined type 2 diabetes mellitus and prognosis in early-stage breast cancer**. (1527-7755 (Electronic)).
97. Kaplan MA, Pekkolay Z Fau - Kucukoner M, Kucukoner M Fau - Inal A, Inal A Fau - Urakci Z, Urakci Z Fau - Ertugrul H, Ertugrul H Fau - Akdogan R, Akdogan R Fau - Firat U, Firat U Fau - Yildiz I, Yildiz I Fau - Isikdogan A, Isikdogan A: **Type 2 diabetes mellitus and prognosis in early stage breast cancer women**. (1559-131X (Electronic)).
98. Peairs KS, Barone Bb Fau - Snyder CF, Snyder Cf Fau - Yeh H-C, Yeh Hc Fau - Stein KB, Stein Kb Fau - Derr RL, Derr Rl Fau - Brancati FL, Brancati Fl Fau - Wolff AC, Wolff AC: **Diabetes mellitus and breast cancer outcomes: a systematic review and meta-analysis**. (1527-7755 (Electronic)).
99. Redaniel MT, Jeffreys M Fau - May MT, May Mt Fau - Ben-Shlomo Y, Ben-Shlomo Y Fau - Martin RM, Martin RM: **Associations of type 2 diabetes and diabetes treatment with breast cancer risk and mortality: a population-based cohort study among British women**. (1573-7225 (Electronic)).
100. Schrauder MG, Fasching Pa Fau - Haberle L, Haberle L Fau - Lux MP, Lux Mp Fau - Rauh C, Rauh C Fau - Hein A, Hein A Fau - Bayer CM, Bayer Cm Fau - Heusinger K, Heusinger K Fau - Hartmann A, Hartmann A Fau - Strehl JD, Strehl Jd Fau - Wachter DL *et al*: **Diabetes and prognosis in a breast cancer cohort**. (1432-1335 (Electronic)).
101. Yeh HC, Platz Ea Fau - Wang N-Y, Wang Ny Fau - Visvanathan K, Visvanathan K Fau - Helzlsouer KJ, Helzlsouer Kj Fau - Brancati FL, Brancati FL: **A prospective study of the associations between treated diabetes and cancer outcomes**. (1935-5548 (Electronic)).
102. Lipscombe LL, Goodwin Pj Fau - Zinman B, Zinman B Fau - McLaughlin JR, McLaughlin Jr Fau - Hux JE, Hux JE: **The impact of diabetes on survival following breast cancer**. (0167-6806 (Print)).
103. Liu X, Ji J Fau - Sundquist K, Sundquist K Fau - Sundquist J, Sundquist J Fau - Hemminki K, Hemminki K: **The impact of type 2 diabetes mellitus on cancer-specific survival: a follow-up study in Sweden**. (1097-0142 (Electronic)).

104. Currie CJ, Poole Cd Fau - Evans M, Evans M Fau - Peters JR, Peters Jr Fau - Morgan CL, Morgan CL: **Mortality and other important diabetes-related outcomes with insulin vs other antihyperglycemic therapies in type 2 diabetes**. (1945-7197 (Electronic)).
105. Ioacara S, Guja C, Ionescu-Tirgoviste C, Fica S, Roden M: **Cancer specific mortality in insulin-treated type 2 diabetes patients**. *PLoS ONE* 2014, **9**(3).
106. Rose DP, Vona-Davis L: **The cellular and molecular mechanisms by which insulin influences breast cancer risk and progression**. (1479-6821 (Electronic)).
107. Brange J, Ribel U, Hansen JF, Dodson G, Hansen MT, Havelund S, Melberg SG, Norris F, Norris K, Snel L *et al*: **Monomeric insulins obtained by protein engineering and their medical implications**. *Nature* 1988, **333**(6174):679-682.
108. Dideriksen LH JL, Drejer K: **Carcinogenic effect on female rats after 12 months administration of teh insulin analogue B10 Asp**. *Diabetes* 1992, **41**(143A).
109. Andersson C, Vaag A, Selmer C, Schmiegelow M, Sorensen R, Lindhardsen J, Gislason GH, Kober L, Torp-Pedersen C: **Risk of cancer in patients using glucose-lowering agents: a nationwide cohort study of 3.6 million people**. *BMJ Open* 2012, **2**(3).
110. Eckardt K, May C, Koenen M, Eckel J: **IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts**. *Diabetologia* 2007, **50**(12):2534-2543.
111. Chisalita SI, Arnqvist HJ: **Insulin-like growth factor I receptors are more abundant than insulin receptors in human micro- and macrovascular endothelial cells**. *Am J Physiol Endocrinol Metab* 2004, **286**(6):E896-901.
112. Kohn WD, Micanovic R, Myers SL, Vick AM, Kahl SD, Zhang L, Strifler BA, Li S, Shang J, Beals JM *et al*: **pI-shifted insulin analogs with extended in vivo time action and favorable receptor selectivity**. *Peptides* 2007, **28**(4):935-948.
113. Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C, Trub T: **Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use**. *Diabetes* 2000, **49**(6):999-1005.
114. Sommerfeld MR, Muller G, Tschank G, Seipke G, Habermann P, Kurrle R, Tennagels N: **In vitro metabolic and mitogenic signaling of insulin glargine and its metabolites**. *PLoS One* 2010, **5**(3):e9540.
115. Warnken M, Reitzenstein U, Sommer A, Fuhrmann M, Mayer P, Enzmann H, Juergens UR, Racke K: **Characterization of proliferative effects of insulin, insulin analogues and insulin-like growth factor-1 (IGF-1) in human lung fibroblasts**. *Naunyn Schmiedebergs Arch Pharmacol* 2010, **382**(5-6):511-524.
116. Weinstein D, Simon M, Yehezkel E, Laron Z, Werner H: **Insulin analogues display IGF-I-like mitogenic and anti-apoptotic activities in cultured cancer cells**. *Diabetes Metab Res Rev* 2009, **25**(1):41-49.
117. Yehezkel E, Weinstein D, Simon M, Sarfstein R, Laron Z, Werner H: **Long-acting insulin analogues elicit atypical signalling events mediated by the insulin receptor and insulin-like growth factor-I receptor**. *Diabetologia* 2010, **53**(12):2667-2675.
118. Staiger K, Hennige AM, Staiger H, Haring HU, Kellerer M: **Comparison of the mitogenic potency of regular human insulin and its analogue glargine in normal and transformed human breast epithelial cells**. *Horm Metab Res* 2007, **39**(1):65-67.
119. Bahr M, Kolter T, Seipke G, Eckel J: **Growth promoting and metabolic activity of the human insulin analogue [GlyA21,ArgB31,ArgB32]insulin (HOE 901) in muscle cells**. *Eur J Pharmacol* 1997, **320**(2-3):259-265.
120. Ciaraldi TP, Carter L, Seipke G, Mudaliar S, Henry RR: **Effects of the long-acting insulin analog insulin glargine on cultured human skeletal muscle cells: comparisons to insulin and IGF-I**. *J Clin Endocrinol Metab* 2001, **86**(12):5838-5847.
121. Liefvendahl E, Arnqvist HJ: **Mitogenic effect of the insulin analogue glargine in malignant cells in comparison with insulin and IGF-I**. *Horm Metab Res* 2008, **40**(6):369-374.
122. Muller K, Weidinger C, Fuhrer D: **Insulin glargine and insulin have identical effects on proliferation and phosphatidylinositol 3-kinase/AKT signalling in rat thyrocytes and human follicular thyroid cancer cells**. *Diabetologia* 2010, **53**(5):1001-1003.
123. Wada T, Azegami M, Sugiyama M, Tsuneki H, Sasaoka T: **Characteristics of signalling properties mediated by long-acting insulin analogue glargine and detemir in target cells of insulin**. *Diabetes Res Clin Pract* 2008, **81**(3):269-277.
124. Erbel S, Reers C, Eckstein VW, Kleeff J, Buchler MW, Nawroth PP, Ritzel RA: **Proliferation of colo-357 pancreatic carcinoma cells and survival of patients with pancreatic carcinoma are not altered by insulin glargine**. *Diabetes Care* 2008, **31**(6):1105-1111.

125. Liu S, Li Y, Lin T, Fan X, Liang Y, Heemann U: **High dose human insulin and insulin glargine promote T24 bladder cancer cell proliferation via PI3K-independent activation of Akt**. *Diabetes Res Clin Pract* 2011, **91**(2):177-182.
126. Liu SY, Liang Y, Lin TX, Su F, Liang WW, Uwe H, Li Y: **MEK1 and MEK2 differentially regulate human insulin- and insulin glargine-induced human bladder cancer T24 cell proliferation**. *Chin Med J (Engl)* 2012, **125**(23):4197-4201.
127. Sciacca L, Cassarino MF, Genua M, Pandini G, Le Moli R, Squatrito S, Vigneri R: **Insulin analogues differently activate insulin receptor isoforms and post-receptor signalling**. *Diabetologia* 2010, **53**(8):1743-1753.
128. Swift S, Lorens J, Achacoso P, Nolan GP: **Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems**. *Curr Protoc Immunol* 2001, **Chapter 10**:Unit 10 17C.
129. Zhang Y, Moerkens M, Ramaiahgari S, de Bont H, Price L, Meerman J, van de Water B: **Elevated insulin-like growth factor 1 receptor signaling induces antiestrogen resistance through the MAPK/ERK and PI3K/Akt signaling routes**. *Breast Cancer Res* 2011, **13**(3):R52.
130. Vichai V, Kirtikara K: **Sulforhodamine B colorimetric assay for cytotoxicity screening**. *Nat Protoc* 2006, **1**(3):1112-1116.
131. Agin A, Jeandidier N, Gasser F, Grucker D, Sapin R: **Glargine blood biotransformation: in vitro appraisal with human insulin immunoassay**. *Diabetes Metab* 2007, **33**(3):205-212.
132. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schanzer W: **Qualitative determination of synthetic analogues of insulin in human plasma by immunoaffinity purification and liquid chromatography-tandem mass spectrometry for doping control purposes**. *Anal Chem* 2005, **77**(11):3579-3585.
133. Hudelist G, Wagner T, Rosner M, Fink-Retter A, Gschwantler-Kaulich D, Czerwenka K, Kroiss R, Tea M, Pischinger K, Kostler WJ *et al*: **Intratumoral IGF-I protein expression is selectively upregulated in breast cancer patients with BRCA1/2 mutations**. *Endocr Relat Cancer* 2007, **14**(4):1053-1062.
134. Vella V, Pandini G, Sciacca L, Mineo R, Vigneri R, Pezzino V, Belfiore A: **A novel autocrine loop involving IGF-II and the insulin receptor isoform-A stimulates growth of thyroid cancer**. *J Clin Endocrinol Metab* 2002, **87**(1):245-254.
135. Belfiore A, Malaguarnera R: **Insulin receptor and cancer**. *Endocr Relat Cancer* 2011, **18**(4):R125-147.
136. Pollak M: **The insulin and insulin-like growth factor receptor family in neoplasia: an update**. *Nat Rev Cancer* 2012, **12**(3):159-169.
137. Owens DR, Bolli GB: **Beyond the era of NPH insulin--long-acting insulin analogs: chemistry, comparative pharmacology, and clinical application**. *Diabetes Technol Ther* 2008, **10**(5):333-349.
138. Duckworth WC, Bennett RG, Hamel FG: **Insulin degradation: progress and potential**. *Endocr Rev* 1998, **19**(5):608-624.
139. Fawcett J, Tsui BT, Kruer MC, Duckworth WC: **Reduced action of insulin glargine on protein and lipid metabolism: possible relationship to cellular hormone metabolism**. *Metabolism* 2004, **53**(8):1037-1044.
140. Lucidi P, Porcellati F, Rossetti P, Candeloro P, Andreoli AM, Cioli P, Hahn A, Schmidt R, Bolli GB, Fanelli CG: **Metabolism of insulin glargine after repeated daily subcutaneous injections in subjects with type 2 diabetes**. *Diabetes Care* 2012, **35**(12):2647-2649.
141. Varewijck AJ, Yki-Jarvinen H, Schmidt R, Tennagels N, Janssen JA: **Concentrations of Insulin Glargine and Its Metabolites During Long-Term Insulin Therapy in Type 2 Diabetic Patients and Comparison of Effects of Insulin Glargine, Its Metabolites, IGF-I, and Human Insulin on Insulin and IGF-I Receptor Signaling**. *Diabetes* 2013.
142. Shi Y, Hu FB: **The global implications of diabetes and cancer**. *Lancet* 2014, **383**(9933):1947-1948.
143. Ljung R, Talback M, Haglund B, Jonasson JM, Gudbjornsdottir S, Steineck G: **Insulin glargine use and short-term incidence of malignancies - a three-year population-based observation**. *Acta Oncol* 2011, **50**(5):685-693.
144. Habel LA, Danforth KN, Quesenberry CP, Capra A, Van Den Eeden SK, Weiss NS, Ferrara A: **Cohort study of insulin glargine and risk of breast, prostate, and colorectal cancer among patients with diabetes**. *Diabetes Care* 2013, **36**(12):3953-3960.
145. Rostoker R, Bitton-Worms K, Caspi A, Shen-Orr Z, LeRoith D: **Investigating new therapeutic strategies targeting hyperinsulinemia's mitogenic effects in a female mouse breast cancer model**. *Endocrinology* 2013, **154**(5):1701-1710.
146. Bordeleau L, Gerstein HC: **Response to Zanders et al. The association of basal insulin glargine and/or n-3 fatty acids with incident cancers in patients with dysglycemia. Diabetes Care 2014;37:1360-1366**. *Diabetes Care* 2014, **37**(10):e223.
147. Hansen BF: **Insulin analogues with increased mitogenic potency--are they safe?** *Horm Metab Res* 2008, **40**(6):431-433.

148. Pollak M: **Insulin and insulin-like growth factor signalling in neoplasia**. *Nat Rev Cancer* 2008, **8**(12):915-928.
149. Hansen BF, Danielsen GM, Drejer K, Sorensen AR, Wiberg FC, Klein HH, Lundemose AG: **Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency**. *Biochem J* 1996, **315 (Pt 1)**:271-279.
150. Romer M, Eichner J, Metzger U, Templin MF, Plummer S, Ellinger-Ziegelbauer H, Zell A: **Cross-platform toxicogenomics for the prediction of non-genotoxic hepatocarcinogenesis in rat**. *PLoS One* 2014, **9**(5):e97640.
151. Melis JP, Derks KW, Pronk TE, Wackers P, Schaap MM, Zwart E, van Ijcken WF, Jonker MJ, Breit TM, Pothof J *et al*: **In vivo murine hepatic microRNA and mRNA expression signatures predicting the (non-)genotoxic carcinogenic potential of chemicals**. *Arch Toxicol* 2014, **88**(4):1023-1034.
152. Huijts PE, van Dongen M, de Goeij MC, van Moolenbroek AJ, Blanken F, Vreeswijk MP, de Kruijf EM, Mesker WE, van Zwet EW, Tollenaar RA *et al*: **Allele-specific regulation of FGFR2 expression is cell type-dependent and may increase breast cancer risk through a paracrine stimulus involving FGF10**. *Breast Cancer Res* 2011, **13**(4):R72.
153. Ter Braak B, Siezen C, Speksnijder EN, Koedoot E, van Steeg H, Salvatori DC, van de Water B, van der Laan JW: **Mammary gland tumor promotion by chronic administration of IGF1 and the insulin analogue AspB10 in the p53(R270H/+)WAPCre mouse model**. *Breast Cancer Res* 2015, **17**(1):518.
154. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: **Exploration, normalization, and summaries of high density oligonucleotide array probe level data**. *Biostatistics (Oxford, England)* 2003, **4**(2):249-264.
155. Wolfinger RD, Gibson G, Wolfinger ED, Bennett L, Hamadeh H, Bushel P, Afshari C, Paules RS: **Assessing gene significance from cDNA microarray expression data via mixed models**. *Journal of computational biology : a journal of computational molecular cell biology* 2001, **8**(6):625-637.
156. Hochberg Y, Benjamini Y: **More powerful procedures for multiple significance testing**. *Statistics in medicine* 1990, **9**(7):811-818.
157. Smyth GK, Michaud J, Scott HS: **Use of within-array replicate spots for assessing differential expression in microarray experiments**. *Bioinformatics (Oxford, England)* 2005, **21**(9):2067-2075.
158. Clark AR, Toker A: **Signalling specificity in the Akt pathway in breast cancer**. *Biochemical Society transactions* 2014, **42**(5):1349-1355.
159. Myung DS, Park YL, Kim N, Chung CY, Park HC, Kim JS, Cho SB, Lee WS, Lee JH, Joo YE: **Expression of early growth response-1 in colorectal cancer and its relation to tumor cell proliferation and apoptosis**. *Oncology reports* 2014, **31**(2):788-794.
160. Chandra A, Lan S, Zhu J, Siclari VA, Qin L: **Epidermal growth factor receptor (EGFR) signaling promotes proliferation and survival in osteoprogenitors by increasing early growth response 2 (EGR2) expression**. *The Journal of biological chemistry* 2013, **288**(28):20488-20498.
161. Suzuki T, Inoue A, Miki Y, Moriya T, Akahira J, Ishida T, Hirakawa H, Yamaguchi Y, Hayashi S, Sasano H: **Early growth responsive gene 3 in human breast carcinoma: a regulator of estrogen-meditated invasion and a potent prognostic factor**. *Endocr Relat Cancer* 2007, **14**(2):279-292.
162. Matsuo T, Dat le T, Komatsu M, Yoshimaru T, Daizumoto K, Sone S, Nishioka Y, Katagiri T: **Early Growth Response 4 Is Involved in Cell Proliferation of Small Cell Lung Cancer through Transcriptional Activation of Its Downstream Genes**. *PLoS One* 2014, **9**(11):e113606.
163. Verset L, Tommelein J, Moles Lopez X, Decaestecker C, Mareel M, Bracke M, Salmon I, De Wever O, Demetter P: **Epithelial expression of FHL2 is negatively associated with metastasis-free and overall survival in colorectal cancer**. *Br J Cancer* 2013, **109**(1):114-120.
164. Sakthianandeswaren A, Christie M, D'Andreti C, Tsui C, Jorissen RN, Li S, Fleming NI, Gibbs P, Lipton L, Malaterre J *et al*: **PHLDA1 expression marks the putative epithelial stem cells and contributes to intestinal tumorigenesis**. *Cancer Res* 2011, **71**(10):3709-3719.
165. Uekusa S, Kawashima H, Sugito K, Yoshizawa S, Shinojima Y, Igarashi J, Ghosh S, Wang X, Fujiwara K, Ikeda T *et al*: **Nr4a3, a possibile oncogenic factor for neuroblastoma associated with CpGi methylation within the third exon**. *Int J Oncol* 2014, **44**(5):1669-1677.
166. Wells JE, Howlett M, Cole CH, Kees UR: **Deregulated expression of connective tissue growth factor (CTGF/CCN2) is linked to poor outcome in human cancer**. *Int J Cancer* 2014.
167. Bechara EG, Sebestyen E, Bernardis I, Eyras E, Valcarcel J: **RBM5, 6, and 10 differentially regulate NUMB alternative splicing to control cancer cell proliferation**. *Molecular cell* 2013, **52**(5):720-733.
168. Yousefzadeh MJ, Wyatt DW, Takata K, Mu Y, Hensley SC, Tomida J, Bylund GO, Doublie S, Johansson E, Ramsden DA *et al*: **Mechanism of suppression of chromosomal instability by DNA polymerase POLQ**. *PLoS genetics* 2014, **10**(10):e1004654.

169. Tennagels N, Werner U: **The metabolic and mitogenic properties of basal insulin analogues**. *Arch Physiol Biochem* 2013, **119**(1):1-14.
170. Tamimi RM, Colditz GA, Wang Y, Collins LC, Hu R, Rosner B, Irie HY, Connolly JL, Schnitt SJ: **Expression of IGF1R in normal breast tissue and subsequent risk of breast cancer**. *Breast Cancer Res Treat* 2011, **128**(1):243-250.
171. Arnaldez FI, Helman LJ: **Targeting the insulin growth factor receptor 1**. *Hematol Oncol Clin North Am* 2012, **26**(3):527-542, vii-viii.
172. Kellerer M, Haring HU: **Insulin analogues: impact of cell model characteristics on results and conclusions regarding mitogenic properties**. *Exp Clin Endocrinol Diabetes* 2001, **109**(1):63-64.
173. Tennagels N, Welte S, Hofmann M, Brenk P, Schmidt R, Werner U: **Differences in metabolic and mitogenic signalling of insulin glargine and insulin aspart B10 in rats**. *Diabetologia* 2013.
174. Stammberger I, Bube A, Durchfeld-Meyer B, Donaubauer H, Troschau G: **Evaluation of the carcinogenic potential of insulin glargine (LANTUS) in rats and mice**. *Int J Toxicol* 2002, **21**(3):171-179.
175. Stammberger I, Essermeant L: **Insulin glargine: a reevaluation of rodent carcinogenicity findings**. *Int J Toxicol* 2012, **31**(2):137-142.
176. Nagel JM, Staffa J, Renner-Muller I, Horst D, Vogeser M, Langkamp M, Hoeflich A, Goke B, Kolligs FT, Mantzoros CS: **Insulin glargine and NPH insulin increase to a similar degree epithelial cell proliferation and aberrant crypt foci formation in colons of diabetic mice**. *Horm Cancer* 2010, **1**(6):320-330.
177. Buchs AE, Silverman BG: **Incidence of malignancies in patients with diabetes mellitus and correlation with treatment modalities in a large Israeli health maintenance organization: a historical cohort study**. *Metabolism* 2011, **60**(10):1379-1385.
178. Fagot JP, Blotiere PO, Ricordeau P, Weill A, Alla F, Allemand H: **Does insulin glargine increase the risk of cancer compared with other basal insulins?: A French nationwide cohort study based on national administrative databases**. *Diabetes Care* 2013, **36**(2):294-301.
179. van Staa TP, Patel D, Gallagher AM, de Bruin ML: **Glucose-lowering agents and the patterns of risk for cancer: a study with the General Practice Research Database and secondary care data**. *Diabetologia* 2012, **55**(3):654-665.
180. Scheer N, Snaith M, Wolf CR, Seibler J: **Generation and utility of genetically humanized mouse models**. *Drug Discov Today* 2013, **18**(23-24):1200-1211.
181. Frese KK, Tuveson DA: **Maximizing mouse cancer models**. *Nat Rev Cancer* 2007, **7**(9):645-658.
182. Wijnhoven SW, Zwart E, Speksnijder EN, Beems RB, Olive KP, Tuveson DA, Jonkers J, Schaap MM, van den Berg J, Jacks T *et al*: **Mice expressing a mammary gland-specific R270H mutation in the p53 tumor suppressor gene mimic human breast cancer development**. *Cancer Res* 2005, **65**(18):8166-8173.
183. Wijnhoven SW, Speksnijder EN, Liu X, Zwart E, vanOostrom CT, Beems RB, Hoogervorst EM, Schaap MM, Attardi LD, Jacks T *et al*: **Dominant-negative but not gain-of-function effects of a p53.R270H mutation in mouse epithelium tissue after DNA damage**. *Cancer Res* 2007, **67**(10):4648-4656.
184. Derksen PW, Braumuller TM, van der Burg E, Hornsveld M, Mesman E, Wesseling J, Krimpenfort P, Jonkers J: **Mammary-specific inactivation of E-cadherin and p53 impairs functional gland development and leads to pleomorphic invasive lobular carcinoma in mice**. *Dis Model Mech* 2011, **4**(3):347-358.
185. Chignola R, Foroni RI: **Estimating the growth kinetics of experimental tumors from as few as two determinations of tumor size: implications for clinical oncology**. *IEEE Trans Biomed Eng* 2005, **52**(5):808-815.
186. Cardiff RD, Anver MR, Gusterson BA, Hennighausen L, Jensen RA, Merino MJ, Rehm S, Russo J, Tavassoli FA, Wakefield LM *et al*: **The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting**. *Oncogene* 2000, **19**(8):968-988.
187. Cardiff RD: **The pathology of EMT in mouse mammary tumorigenesis**. *J Mammary Gland Biol Neoplasia* 2010, **15**(2):225-233.
188. Radaelli E, Arnold A, Papanikolaou A, Garcia-Fernandez RA, Mattiello S, Scanziani E, Cardiff RD: **Mammary tumor phenotypes in wild-type aging female FVB/N mice with pituitary prolactinomas**. *Vet Pathol* 2009, **46**(4):736-745.
189. Hvid H, Blouin MJ, Birman E, Damgaard J, Poulsen F, Fels JJ, Fledelius C, Hansen BF, Pollak M: **Treatment with insulin analog X10 and IGF-1 increases growth of colon cancer allografts**. *PLoS One* 2013, **8**(11):e79710.
190. Klarenbeek S, van Miltenburg MH, Jonkers J: **Genetically engineered mouse models of PI3K signaling in breast cancer**. *Mol Oncol* 2013, **7**(2):146-164.
191. Varewijck AJ, Janssen JA: **Insulin and its analogs and their affinities to the IGF-1 receptor**. *Endocr Relat Cancer* 2012.

192. Chang CH, Toh S, Lin JW, Chen ST, Kuo CW, Chuang LM, Lai MS: **Cancer risk associated with insulin glargine among adult type 2 diabetes patients--a nationwide cohort study**. *PLoS One* 2011, **6**(6):e21368.
193. Sciacca L, Cassarino MF, Genua M, Vigneri P, Giovanna Pennisi M, Malandrino P, Squatrito S, Pezzino V, Vigneri R: **Biological effects of insulin and its analogs on cancer cells with different insulin family receptor expression**. *Journal of cellular physiology* 2014, **229**(11):1817-1821.
194. Ter Braak B SC, Speksnijder EN, Koedoot E, Van Steeg H, Salvatori DCF, Van de Water B and Van der Laan JW: **Mammary gland tumor promotion by chronic administration of IGF1 and the insulin analogue AspB10 in the p53R270H/+WAPCre mouse model**. *Breast Cancer Res* 2015.
195. Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, Crowley D, Jacks T: **Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome**. *Cell* 2004, **119**(6):847-860.
196. Torsoli A: **Sanofi Accelerates Development of New Lantus Formulation**. In.: bloomberg; 2011.
197. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation**. *Cell* 2011, **144**(5):646-674.
198. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., Kinzler KW: **Cancer genome landscapes**. *Science* 2013, **339**(6127):1546-1558.
199. Yizhak K, Le Devedec SE, Rogkoti VM, Baenke F, de Boer VC, Frezza C, Schulze A, van de Water B, Ruppin E: **A computational study of the Warburg effect identifies metabolic targets inhibiting cancer migration**. *Molecular systems biology* 2014, **10**:744.
200. Chang C-C, Lin C-J: **LIBSVM: A library for support vector machines**. *ACM Trans Intell Syst Technol* 2011, **2**(3):1-27.
201. Zur H, Ruppin E, Shlomi T: **iMAT: an integrative metabolic analysis tool**. *Bioinformatics (Oxford, England)* 2010, **26**(24):3140-3142.
202. Duarte NC, Becker SA, Jamshidi N, Thiele I, Mo ML, Vo TD, Srivas R, Palsson BO: **Global reconstruction of the human metabolic network based on genomic and bibliomic data**. *Proc Natl Acad Sci U S A* 2007, **104**(6):1777-1782.
203. Crea F, Hurt EM, Mathews LA, Cabarcas SM, Sun L, Marquez VE, Danesi R, Farrar WL: **Pharmacologic disruption of Polycomb Repressive Complex 2 inhibits tumorigenicity and tumor progression in prostate cancer**. *Mol Cancer* 2011, **10**:40.
204. Bae WK, Hennighausen L: **Canonical and non-canonical roles of the histone methyltransferase EZH2 in mammary development and cancer**. *Mol Cell Endocrinol* 2014, **382**(1):593-597.
205. Kai K, Iwamoto T, Kobayashi T, Arima Y, Takamoto Y, Ohnishi N, Bartholomeusz C, Horii R, Akiyama F, Hortobagyi GN *et al*: **Ink4a/Arf(-/-) and HRAS(G12V) transform mouse mammary cells into triple-negative breast cancer containing tumorigenic CD49f(-) quiescent cells**. *Oncogene* 2014, **33**(4):440-448.
206. Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A, Vigneri R: **Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells**. *Mol Cell Biol* 1999, **19**(5):3278-3288.
207. Gallagher EJ, Alikhani N, Tobin-Hess A, Blank J, Buffin NJ, Zelenko Z, Tennagels N, Werner U, Leroith D: **Insulin receptor phosphorylation by endogenous insulin or the insulin analog AspB10 promotes mammary tumor growth independent of the IGF-1 receptor**. *Diabetes* 2013.
208. Golpour M, Akhavan Niaki H, Khorasani HR, Hajian A, Mehrasa R, Mostafazadeh A: **Human fibroblast switches to anaerobic metabolic pathway in response to serum starvation: a mimic of warburg effect**. *International journal of molecular and cellular medicine* 2014, **3**(2):74-80.
209. Rader RA: **(Re)defining biopharmaceutical**. *Nature biotechnology* 2008, **26**(7):743-751.
210. ICHS1A: **Guideline on the need for carcinogenicity studies of Pharmaceuticals**. In.; 1995.
211. Sciacca L, Le Moli R, Vigneri R: **Insulin analogs and cancer**. *Front Endocrinol (Lausanne)* 2012, **3**:21.
212. Lind M, Fahlen M, Eliasson B, Oden A: **The relationship between the exposure time of insulin glargine and risk of breast and prostate cancer: an observational study of the time-dependent effects of antidiabetic treatments in patients with diabetes**. *Prim Care Diabetes* 2012, **6**(1):53-59.
213. Vigneri P, Frasca F, Sciacca L, Pandini G, Vigneri R: **Diabetes and cancer**. *Endocr Relat Cancer* 2009, **16**(4):1103-1123.
214. Hanahan D, Weinberg RA: **The hallmarks of cancer**. *Cell* 2000, **100**(1):57-70.
215. van Miltenburg MH, Jonkers J: **Using genetically engineered mouse models to validate candidate cancer genes and test new therapeutic approaches**. *Current opinion in genetics & development* 2012, **22**(1):21-27.
216. Yin M, Zhou J, Gorak EJ, Quddus F: **Metformin is associated with survival benefit in cancer patients with concurrent type 2 diabetes: a systematic review and meta-analysis**. *Oncologist* 2013, **18**(12):1248-1255.

List of abbreviations

◀ IN THE PICTURE

-150 °C freezer. In this PhD-research many different cell lines have been used, some genetically modified and others even have been isolated and generated. Cells can be put in small tubes (with coloured lids) and cryo-preserved for many years in these special freezers. DMSO will prevent the water from forming ice crystals and damaging the cells. Fog coming from the freezer is water vapour condensing due to the drop in temperature.

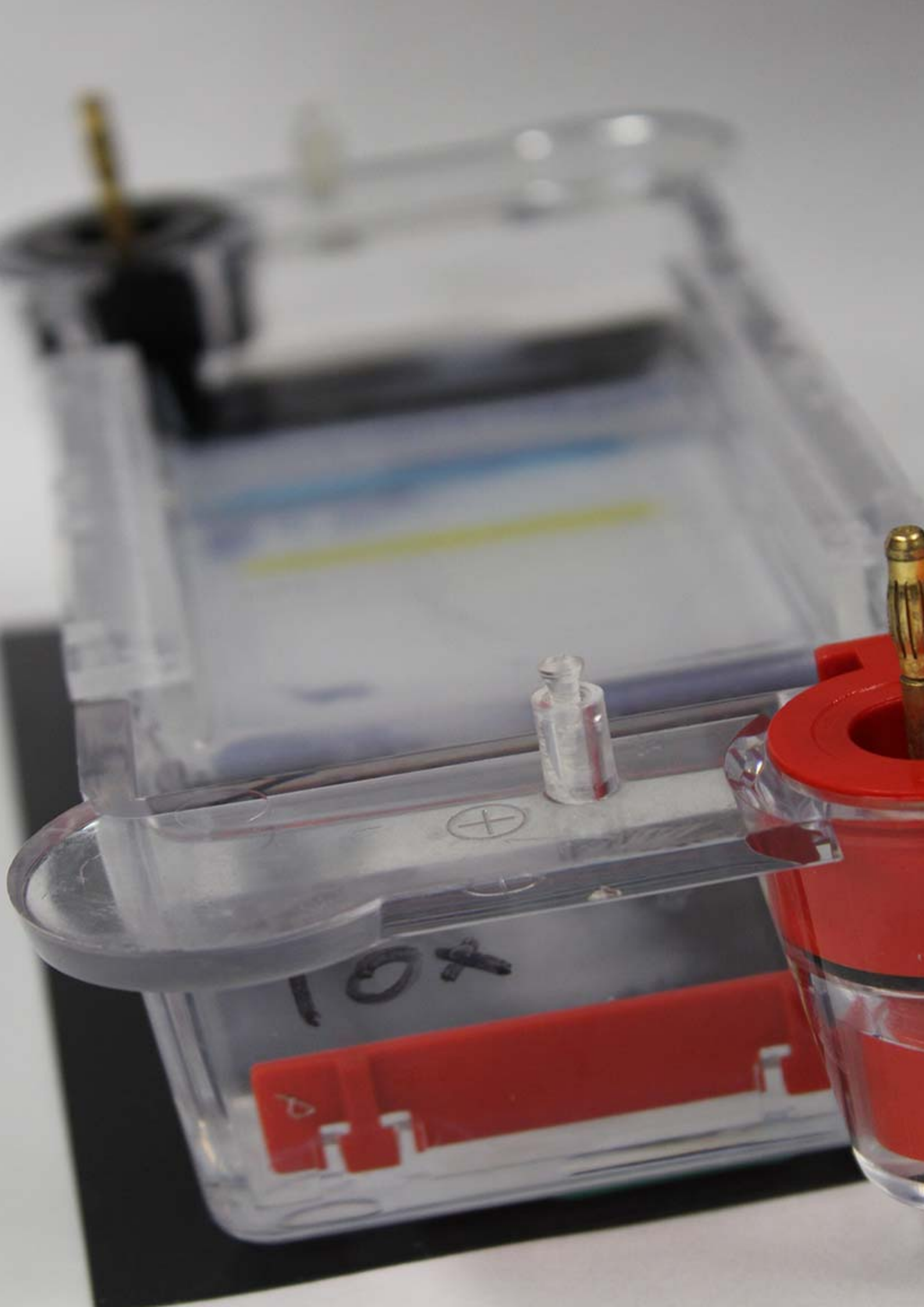
◀ IN BEELD

-150 °C vriezer. Gedurende deze PhD zijn veel verschillende cellijnen gebruikt, genetisch gemodificeerd en anderen zelfs geïsoleerd en gemaakt. Deze cellen kunnen voor jaren in leven worden gehouden door ze in de kleine buisjes (met gekleurde dopjes) te doen en ze heel koud te bewaren. DMSO zorgt ervoor dat er geen waterkristallen gevormd worden die de cellen kunnen beschadigen. De mist die uit de vriezer komt is waterdamp dat direct condenseert.

List of abbreviations

AKT	Serine/threonine kinase
BC	Breast Cancer
BSA	Bovine serum albumin
CDFBS	Charcoal dextran treated FBS
DEG	Differentially expressed gene
DM	Diabetes mellitus
DM1	DM type 1
DM2	DM type 2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	Endogenous control
EC50	Effective concentration 50%
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
ESM	Electronic supplementary material
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDR	False discovery rate
GEM	Genetically engineered mouse
H&E	Hematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
HRP	Horseradish peroxidase
IF	Immunofluorescence
IGF	Insulin like growth factor
IGF1R	IGF-1 receptor
IGFBP	IGF binding protein
IHC	Immuno histo chemistry
INSR	Insulin receptor
IPA®	Ingenuity pathway analysis
IRA	A isoform of IR
IRB	B isoform of IR
IRR	Incidence rate ratio

IRS-1	IR substrate 1
IU	International unit
KD	Knock-down
KM	Kaplan Meier
KO	Knock-out
KS	Kolmogorov-Smirnov
M1	First metabolite of Glargine
M2	Second metabolite of Glargine
MAPK	Mitogen activated protein kinases
MET	Mesenchymal-to-epithelial transition
MeV	Multi-experiment viewer
MG	Mammary gland
MILP	Mixed integer linear programming
MPD	Maximal pharmacological dose
mRNA	messenger RNA
MTD	Maximal tolerance dose
MTLT	Mammary gland tumor latency time
mTOR	Mechanistic target of rapamycin
NGS	Next generation sequencing
NIAD	Non-insulin anti-diabetic drug
NPH	Neutral protamine Hagedorn
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RCT	Randomized controlled trial
RNA	Ribonucleic acid
SD	Standard deviation
SEM	Standard error of mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SRB	Sulforhodamine B
WAP	Whey acidic protein
WB	Western blot
Wt	Wild type
X10	Insulin AspB10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Samenvatting

◀ IN THE PICTURE

DNA is a molecule that encodes the genetic instructions of all living organisms. Small changes in this molecule may have big consequences on biological processes. A scientist can make specific mutations to unravel the function of specific parts (or genes) of this molecule. In the picture, gel electrophoreses equipment is presented. This machine makes use of an electrical current to separate fractions of the DNA molecule. The blue line represents large fragments and in the yellow line smaller fractions are present.

◀ IN BEELD

DNA is een molecuul waarin alle genetische informatie is opgeslagen van alle organismen. Kleine veranderingen in dit molecuul kunnen grote biologische effecten teweeg brengen. Een onderzoeker kan specifieke mutaties maken om de functie van delen van dit molecuul (genoemd 'genen') te achterhalen. Op de foto is een elektroforese apparaat te zien. Met behulp van dit apparaat kunnen stukjes van het DNA molecuul op grootte worden gescheiden. In de blauwe lijn op de foto zitten grote fragmenten van het DNA, in de gele de kleine.

Wereldwijd zijn er momenteel meer dan 380 miljoen diabetes patiënten en door de westerse levenswijze denkt men dat dit aantal de komende jaren fors gaat toenemen. Al deze patiënten zullen uiteindelijk medicijnen moeten gebruiken om hun bloedglucosewaarden op pijl te houden. Een veel gebruikte behandeling is een dagelijks injectieregime van de zogenaamde insuline analogen. Dit zijn moleculen die op moleculair niveau heel veel op normale insuline lijken, maar kleine mutaties hebben grote gevolgen op de werkingsduur van het insuline molecuul. Zo zijn er kort en lang werkende insuline analogen. Insuline glargine (LANTUS) is een voorbeeld van een lang werkend insuline analoog en wordt wereldwijd door miljoenen mensen gebruikt. Drie kleine mutaties in het glargine molecuul hebben ervoor gezorgd dat het na injectie langzaam opgenomen wordt door het menselijk lichaam waardoor het slechts eenmaal per dag gebruikt hoeft te worden door de diabetes patiënt. Deze mutaties hebben er echter ook voor gezorgd dat het molecuul naast een hoge affiniteit voor de insuline receptor (IR) ook een verhoogde affiniteit voor de insulin-like growth factor-1 receptor (IGF1R) heeft. Waar de IR voornamelijk betrokken is bij de metabole effecten (zoals glucose opname) heeft de IGF1R een grote rol in de mitogene effecten (zoals cel deling en groei). Verschillende patiëntenstudies hebben inderdaad de link gelegd tussen het gebruik van glargine en een hogere kans op het krijgen van kanker, met name borstkanker, terwijl andere studies dit effect niet hebben gevonden. De interpretatie van dergelijke patiëntenstudiespatiëntenstudies is vaak erg lastig omdat diabetes zelf al een verhoogd risico vormt voor het ontwikkelen van kanker. Daarnaast worden vaak belangrijke patiënten gegevens (leeftijd, geslacht, leefgewoonten, duur en dosis van insuline behandeling) niet meegenomen in de interpretatie van deze studies terwijl deze factoren grote effecten kunnen hebben op het risico op het ontwikkelen van kanker. Een studie waarbij de invloed van deze mogelijke factoren wordt uitgesloten, en waar slechts de insulinebehandeling verschilt, zou deze tekortkomingen verhelpen. Helaas zijn dergelijke studies schaars en omvatten tot nu toe niet genoeg patiënten. Het directe mitogene effect van deze moleculen zou ook bepaalt kunnen worden met in vitro experimenten waarbij het aantal celdelingen wordt gekwantificeerd door gebruik te maken van cellijnen. Er zijn momenteel ongeveer 50 studies in de literatuur bekend die dergelijke experimenten beschrijven. De resultaten van deze in vitro studies zijn daarnaast vaak tegenstrijdig, voornamelijk omdat ze een verschillende methodologie gebruiken. Daarnaast kunnen enkele parameters niet bestudeerd worden door middel van dergelijke studies. Om de kankerverwekkende eigenschappen van insuline analogen in een levend wezen te bepalen is het helaas onvermijdelijk in vivo experimenten uit te voeren. Bij dergelijke experimenten worden dieren, in veel gevallen muizen of ratten, geïnjecteerd met een hoge dosis van een bepaalde stof. Vervolgens wordt gekeken of bij deze dieren de tumor eigenschappen (incidentie, type, grote etc.) beïnvloed zijn. Studies die dieren chronisch hebben blootgesteld aan insuline analogen zijn in aantal zeer gering maar hebben er wel toe geleid dat een bepaalde insuline analoog, AspB10, nooit op de markt is gekomen omdat er een verhoogde tumor incidentie werd waargenomen.

De hoge kosten van dergelijke studies is zeer waarschijnlijk de reden van deze schaarste. De organisatie die verantwoordelijk is voor de toelating van nieuwe medicijnen op de Europese markt, EMA, heeft onlangs aangegeven hoe men beter en doelgerichter onderzoek kan doen naar de kankerverwekkende eigenschappen van insuline analogen. In deze thesis is beschreven hoe wij, gebaseerd op dit document, gedurende 4 jaar onderzoek hebben gedaan naar deze stoffen.

Om iets nieuws te onderzoeken met men eerst weten wat er al is gedaan. Daarom zijn we begonnen met het uitvoeren van een uitgebreid en systematisch literatuuronderzoek, welke beschreven staat in **hoofdstuk 2**. In dit review hebben we resultaten van zowel patiëntenstudies, *in vitro*- als *in vivo*- studies opgenomen en hebben we ons gericht op de blootstelling aan insuline analogen in relatie tot borstkanker. Hierin kwam naar voren dat studies in veel gevallen slecht zijn opgezet en niet de juiste positieve en negatieve controles hanteren. Daarnaast zijn er niet genoeg in vivo studies uitgevoerd om uitsluitsel te kunnen geven over de mitogene potentiaal van deze moleculen in een levend organisme.

In **hoofdstuk 3** wordt beschreven hoe wij nieuwe cellijnen gegenereerd hebben om de mitogene potentiaal te kunnen bepalen van alle commerciële insuline analogen. Met dit celsysteem was het mogelijk om de betrokkenheid van de verschillende receptoren te kunnen bepalen. Glargine bleek op korte termijn mitogene signaal transducerende cascades te induceren vooral via de IGF1R. Maar dit leidde er niet toe dat op langere termijn veel meer cellen aanwezig waren in vergelijking tot de insuline blootstelling. Uiteindelijk bleek dat glargine in deze langdurige experimenten afgebroken werd in bepaalde moleculen met een lage mitogene potentiaal. Deze bevinding is ook klinisch van groot belang omdat deze omzetting ook in het menselijk lichaam blijkt plaats te vinden.

Hetzelfde celsysteem wordt gebruikt in de experimenten beschreven in **hoofdstuk 4**. Hier hebben we geprobeerd om op transcriptioneel niveau te achterhalen welke genen betrokken zijn bij de verhoogde mitogeniteit van enkele insuline analogen. Vervolgens hebben we de expressie van deze twintig genen gebruikt om de mitogene potentiaal van andere insuline analogen te voorspellen. Het voorspellend vermogen bleek hoog en stabiel in verschillende modellen. We hopen dat dit systeem overgenomen kan worden door de farmaceutische industrie om vroeg in het ontwikkelingsproces de mitogene bijwerkingen van nieuwe medicijnen te ontdekken.

Eerder hebben we aangegeven wat het belang is van het uitvoeren van experimenten waarbij een levend organisme chronisch wordt blootgesteld aan insuline analogen. In **hoofdstuk 5**, beschrijven we een dergelijk experiment. Hierbij hebben we een speciaal muismodel gebruikt met een genetische achtergrond waarbij ze spontaan humaan relevante borsttumoren ontwikkelen. Wij hebben gekeken hoe chronische blootstelling aan insuline analogen verschillende parameters van de tumorontwikkeling beïnvloedt. Zo hebben we kunnen vaststellen dat na frequente AspB10 en IGF1 injectie de tumoren significant eerder ontstonden.

Ook konden we meten dat de mitogene signaaltransductie in deze tumoren verhoogd was. Dit laatste was ook het geval bij enkele tumoren uit de glargine injectie groep.

In het laatste experimentele hoofdstuk, **hoofdstuk 6**, hebben we de tumoren uit het bovengenoemd experiment verder onderzocht. Door gebruik te maken van een nieuwe techniek konden we het gehele transcriptoom van de tumoren onderzoeken. We kwamen erachter dat verschillende parameters die belangrijk worden geacht in de ontwikkeling van een tumor beïnvloed waren door de langdurige insuline analogen (AspB10) blootstelling. Zoals verwacht had chronische blootstelling aan insuline analogen geen direct effect op de stabiliteit van DNA in de tumoren.

Kortom, het onderzoek dat in dit proefschrift beschreven staat, toont aan dat glargine zelf een verhoogde mitogene potentiaal heeft, maar dat er geen directe aanwijzingen zijn dat dit ook leidt tot een verhoogd risico op het ontwikkelen van kanker in een levend organisme. Alle andere commerciële insuline analogen bleken geen verhoogde celdeling te induceren in vergelijking met normaal humaan insuline. Meer onderzoek op dit vlak is zeker nodig en we hopen dat de kennis die we hier hebben opgedaan in de toekomst zal leiden tot het verbeteren van de mitogeniteitstesten door de farmaceutische industrie. Door het gebruik van slimme modellen en een goed doordachte methodologie kan men grote stappen zetten in het kankeronderzoek.

Dankwoord

◀ IN THE PICTURE

In science, and especially in the laboratory of Toxicology, there is a strong trend to scale up the experiments. “If the insulin receptor downstream signalling pathway has 200 targets, why test one if you can test all of them?” It is impossible to perform these high throughput experiments with manual pipetting alone. The liquid handler in the picture can be programmed to pipette 96 individual wells all at once, which saves time and physical problems like a pipetting hand.

◀ IN BEELD

In het onderzoek, en vooral bij de afdeling Toxicologie worden de experimenten steeds groter opgezet. ”Als de insuline receptor pathway 200 moleculen bevat, waarom zou je er maar één testen als je ze ook allemaal zou kunnen testen?”. Het zou onmogelijk zijn om deze grootschalige experimenten allemaal met de hand te pipetteren. De automatische pipeteer robot op de foto kan 96 individuele welletjes tegelijk pipeteren. Qua tijdsinvestering maar ook uit ergonomisch oogpunt is dit gunstiger.

Curiculum Vitae

Bas (Sebastiaan Johannes) ter Braak was born in Zelhem, The Netherlands, on August 11th 1987. He went to the Ulenhof College in Doetinchem, where he obtained his VWO diploma in 2005, with majors in *Natuur en Gezondheid* and *Natuur en Techniek*.

In 2005, he started his study Biotechnology at Wageningen University. During his academic studies, he completed three graduation Master projects. During a thesis project at the Laboratory of Microbiology of this University he studied the reactivation of inactive genes in *Aspergillus niger*. During his internship at the Bioengineering Research Group of Instituto Superior Técnico (Lisbon Technical University, Portugal), supported by the Erasmus grant, he studied the impact of downstream processing of plasmid on transient transfection in mammalian cells. Bas finished his studies with a second Master thesis at the Biology department of O. Wayne Rollins Research Center (Emory University, Atlanta, USA) where he studied the influence of secondary symbionts in the pea aphid immune system, which was supported by the *Middelhovenfonds*.

Directly after completing his studies in September 2010, he started working as a PhD at the Leiden Academic Center for Drug Research at the Leiden University, on the project "Carcinogenicity of insulin analogues". This project was carried out under the supervision of prof. dr. Bob van de Water, Dr. Jan Willem van der Laan and Dr. Kris Siezen. Since May 2015 he is employed as a post-doctoral researcher at the same department on a project in which cell signalling reporter stem cell models are established for the mechanistic understanding of liver disease.

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List of publications

Carcinogenicity of Biophamaceuticals
Dempster M, Siezen K, **Ter Braak B**, van den Brink W, Emerenciana A, Bellanti F, Duijnhoven RG, Kwa M, Van der Laan JW. *In press March 2015,* "Genotoxicity and Carcinogenicity Testing of Pharmaceuticals", Springer Press (book chapter)

Insulin treatment and breast cancer risk; a review of in vitro, animal and human evidence
Ter Braak B[*], Bronsveld HK[*], Karlstad Ø, Vestergaard P, Starup-Linde J, Bazelier MT, De Bruin ML, De Boer A, Siezen CLE, Van de Water B, Van der Laan JW, Schmidt MK
[*] *Both authors contributed equally.* In review (March 2015), Breast Cancer Research

Alternative signalling network activation through different insulin receptor family members caused by pro-mitogenic antidiabetic insulin analogues in human mammary epithelial cells
Ter Braak B, Wink S, Koedoot E, Pont C, Siezen CL, Van der Laan JW, Van de Water B
In review (Februari 2015) Breast Cancer Research

Mammary gland tumor promotion by chronic 3 administration of IGF1 and the insulin analogue AspB10 in the p53$^{R270H/+}$WAPCre mouse model
Ter Braak B, Siezen CLE, Speksnijder EN, Koedoot E, Van Steeg H, Salvatori DCF, Van de Water B and Van der Laan JW
Breast Cancer Research, March 2015, doi:10.1186/s13058-015-0518-y

Tumorigenic insulin analogues promote mammary gland tumor development by increasing glycolysis and promoting biomass production
Ter Braak B, Siezen CLE, Lee J , Rao P , Voorhoeve C , Ruppin E , Van der Laan JW, Van de Water
Manuscript in preparation.

Classifying the adverse mitogenic mode of action of insulin analogues using a novel mechanism-based genetically engineered human breast cancer cell panel.
Ter Braak B, Siezen CL, Kannegieter N, Koedoot E, van de Water B, van der Laan JW.
Arch Toxicol. 2014 Apr;88(4):953-66. doi: 10.1007/s00204-014-1201-2. Epub 2014 Jan 25. PMID: 24464500

Impact of plasmid quality on lipoplex-mediated transfection.
De La Vega J, **Ter Braak B**, Azzoni AR, Monteiro GA, Prazeres DM.
J Pharm Sci. 2013 Nov;102(11):3932-41. doi: 10.1002/jps.23709. Epub 2013 Aug 28. PMID: 23996350

Exposure to bacterial signals does not alter pea aphids' survival upon a second challenge or investment in production of winged offspring.
Ter Braak B, Laughton AM, Altincicek B, Parker BJ, Gerardo NM.
PLoS One. 2013 Aug 29;8(8):e73600. doi: 10.1371/journal.pone.0073600. eCollection 2013. PMID:24009760

Escherichia coli K-12 pathogenicity in the pea aphid, Acyrthosidpon pisum, reveals reduced antibacterial defense in aphids.
Altincicek B, **Ter Braak B**, Laughton AM, Udekwu KI, Gerardo NM.
Dev Comp Immunol. 2011 Oct;35(10):1091-7. doi: 10.1016/j.dci.2011.03.017. Epub 2011 Apr 20. PMID:21527277