# Carcinogenicity of insulin analogues

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# 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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**◀ 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.

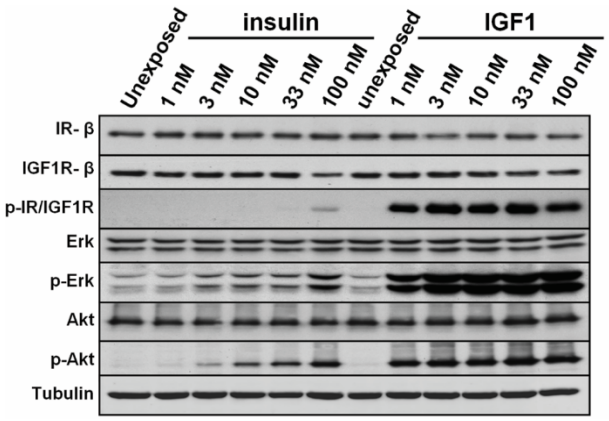


**Fig 1 Dose dependent activation of receptors and downstream signalling pathways in MCF7 cells.** Treatment of MCF-7 cells with either insulin or IGF1 caused a concentration dependent activation of the IR and IGF1R, respectively. MCF-7 was stimulated for 30 minutes with human Insulin and IGF1 (at 1, 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).

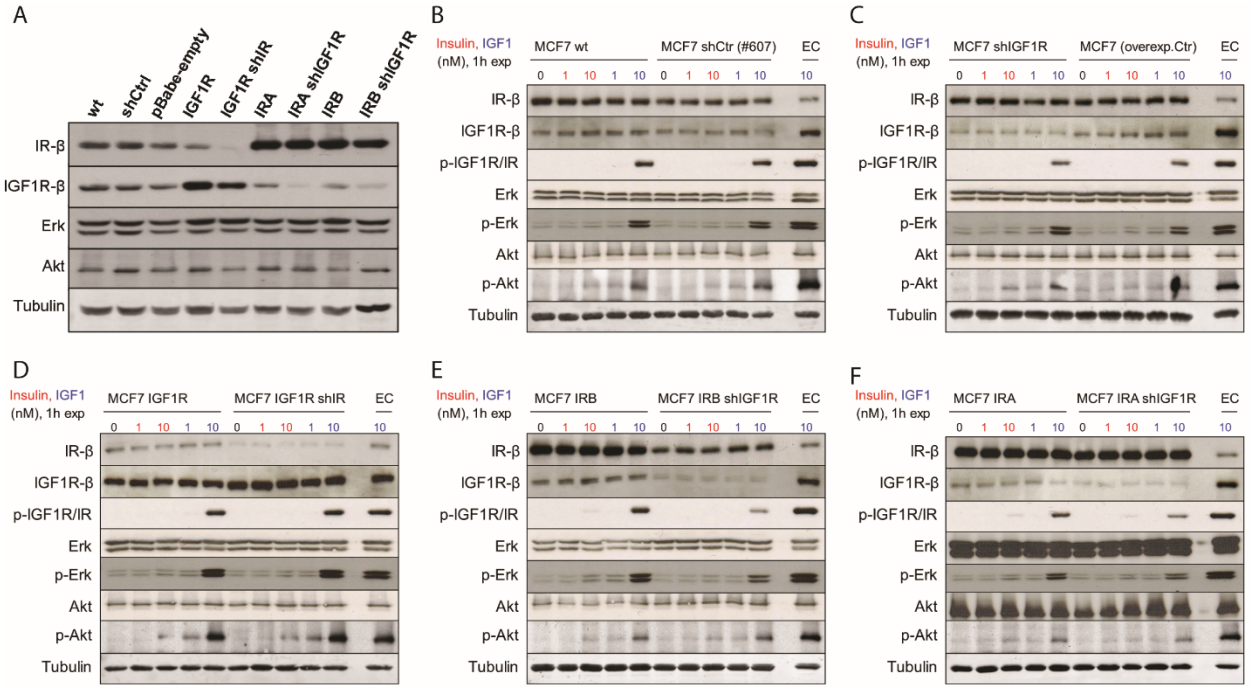


**Fig 2 Generation of a panel of MCF7 cell lines with different receptor levels with an active downstream signalling pathway.** A) Western blot analysis on protein levels (IR, IGF1R, Erk and Akt) of the established cell lines (MCF-7 wt, MCF-7 shCtrl, MCF-7 pBABE-empty, MCF-7 IGF1R, MCF-7 IGF1R shIR, 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.

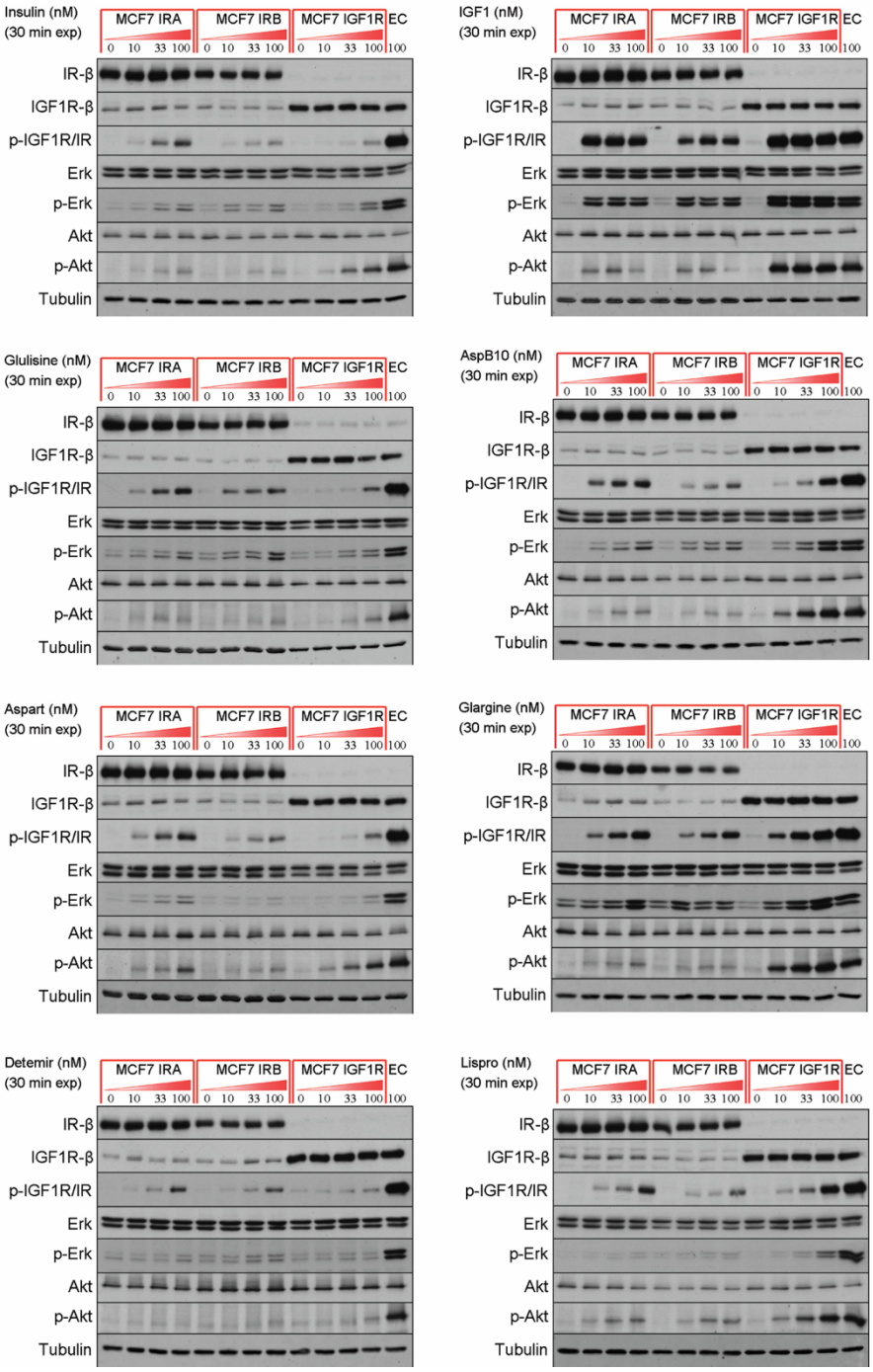


**Fig 3. Stimulation of a panel of MCF7 cell lines with insulin analogues to measure downstream mitogenic signalling.** Westernblot analysis of 3 different cell lines (MCF-7 IRA shIGF1R, MCF-7 IRB shIGF1R and MCF-7 IGF1R shIR) stimulated by 8 different insulin like molecules (human insulin, AspB10, IGF1, 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.

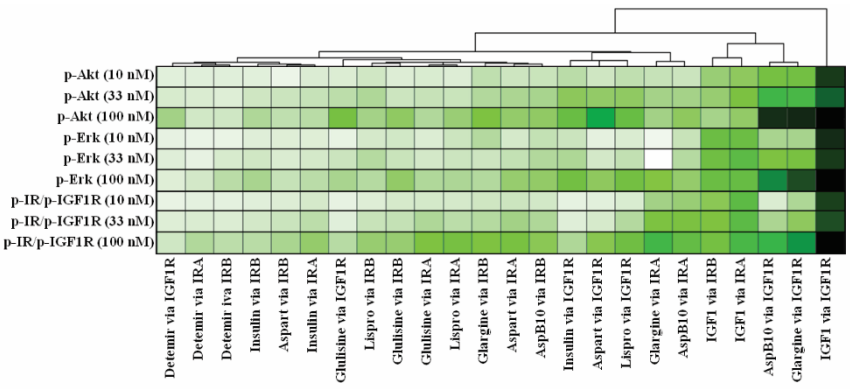


**Fig 4. Hierarchical clustering (using Euclidean distance) of quantified westernblot exposure data**. A White color indicates no difference in activation compared to the unstimulated sample, the increasing dark green color indicates an increasing phosphorylation of either the receptor, p-Erk or p-Akt.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

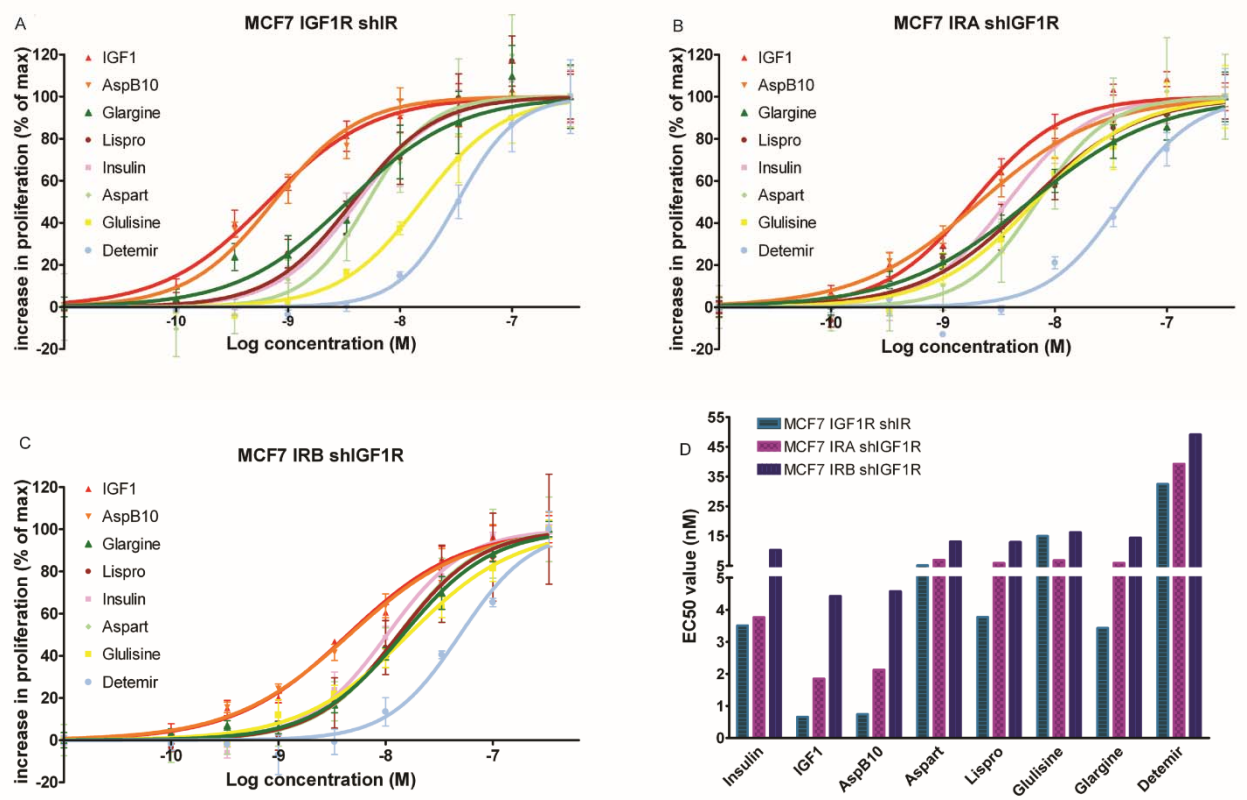


**Fig 5 Dose dependent proliferative effect of all insulin analogues in panel of MCF7 cell lines** a-c Effect of insulin analogues on MCF7 cell proliferation. 3 Cell lines (MCF-7 IGF1R, MCF-7 IRA and MCF-7 IRB) stimulated with 10 different insulin like molecules. Proliferation measured after 4 days of 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.

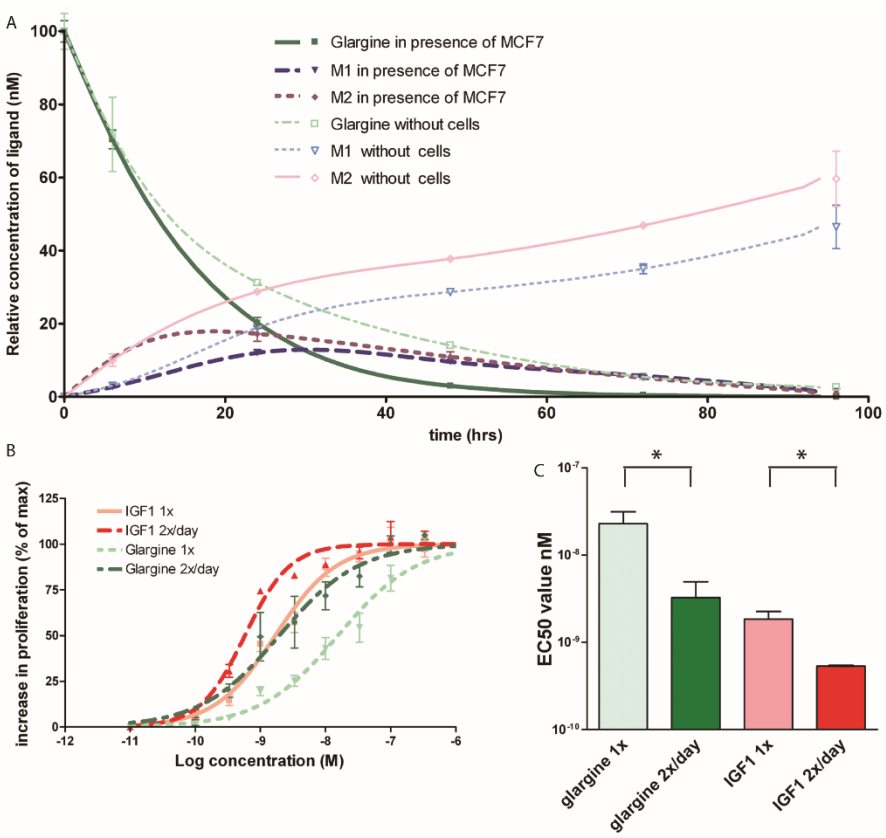


**Fig 6 Glargine metabolism in long term *in vitro* assay** A) Concentrations of insulin glargine, M1 and M2 over time in 5% CDFBS containing RPMI medium in the presence and absence of MCF-7 cells. B) Relative proliferation measured in MCF-7 IGF1R, stimulated once with IGF1 (light red) or glargine (light 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.

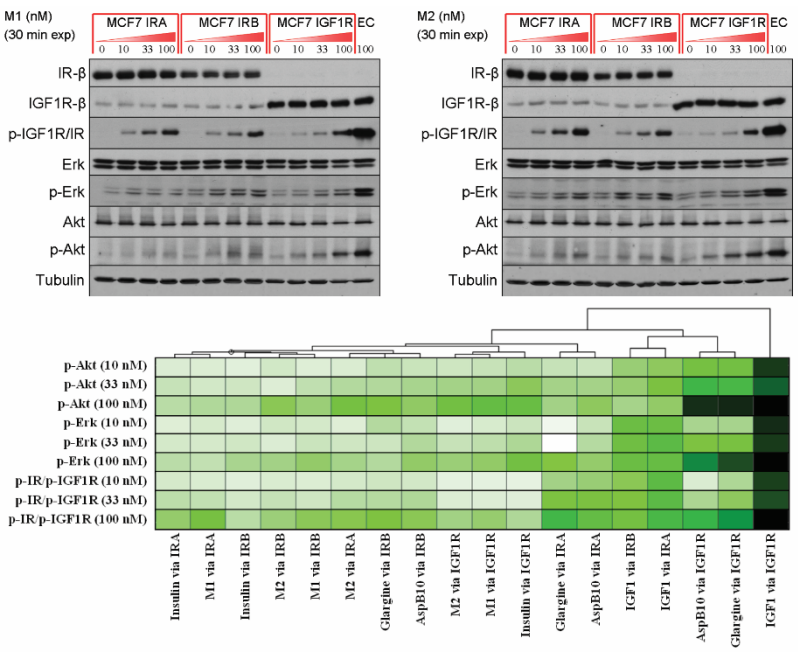


**Fig 7 Metabolites of glargine show mitogenic pathway activation pattern similar to regular insulin.** Westernblot analysis of 3 different cell lines (MCF-7 IRA shIGF1R, MCF-7 IRB shIGF1R and MCF-7 IGF1R shIR) stimulated by M1 and M2 at 4 different concentrations. (n=2) Only blots of the first experiments 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)

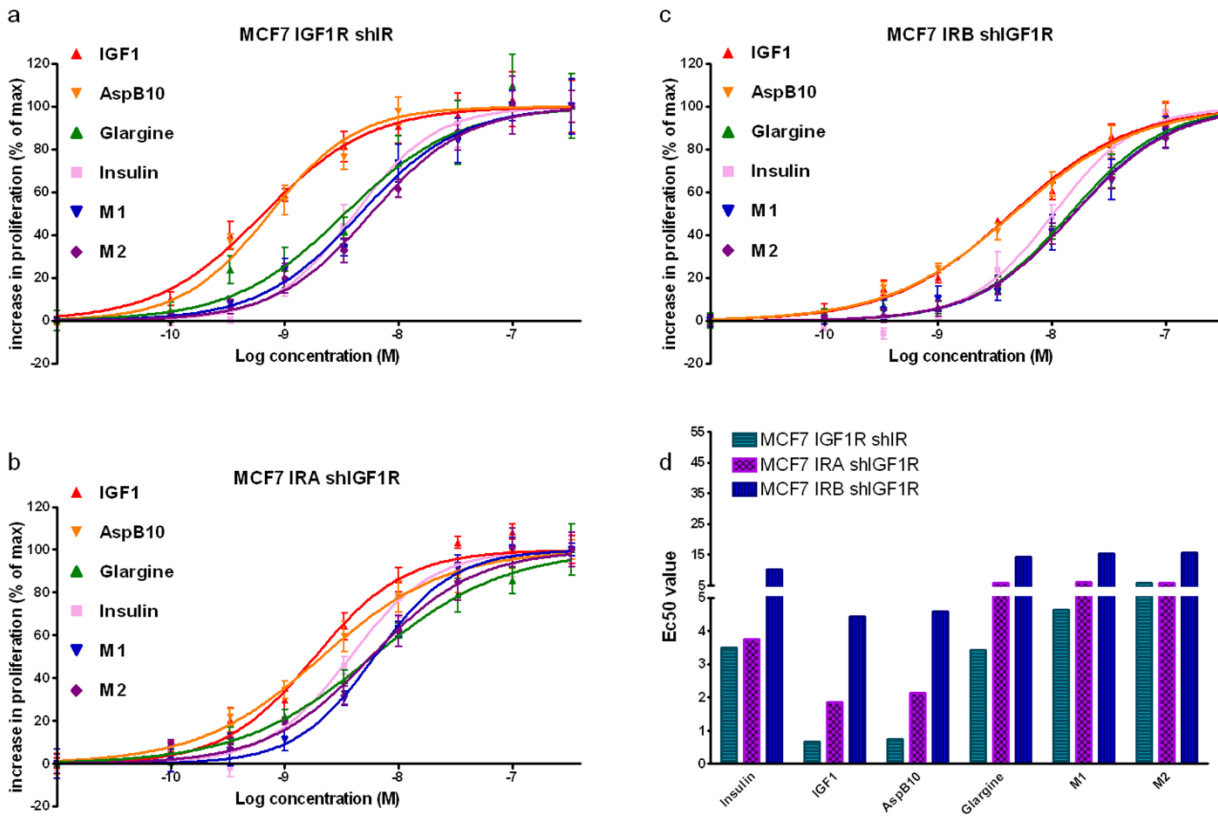


**Fig 8 Metabolites of glargine show similar proliferative potential compared to regular insulin.** a-c Relative proliferation measured for 3 Cell lines (MCF-7 IGF1R, MCF-7 IRA and MCF-7 IRB) stimulated with 6 different insulin like molecules. Proliferation measured after 4 days of stimulation. All values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

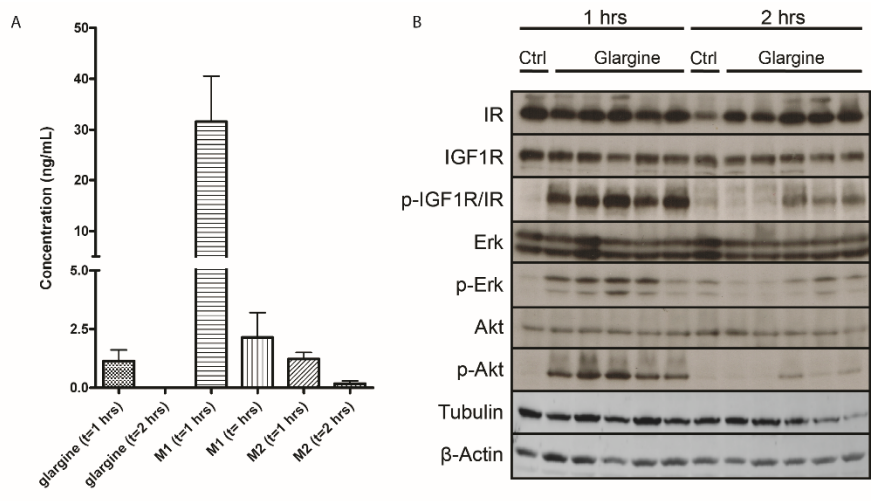


**Fig. 9. Glargine metabolism and mitogenic signalling *in vivo*.** Samples for glargine metabolism were taken at 1 or 2 hours after injection with glargine/vehicle. Activation of Akt and Erk in the mammary gland of mice was detected at 1 and 2 hours after glargine injections.



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.



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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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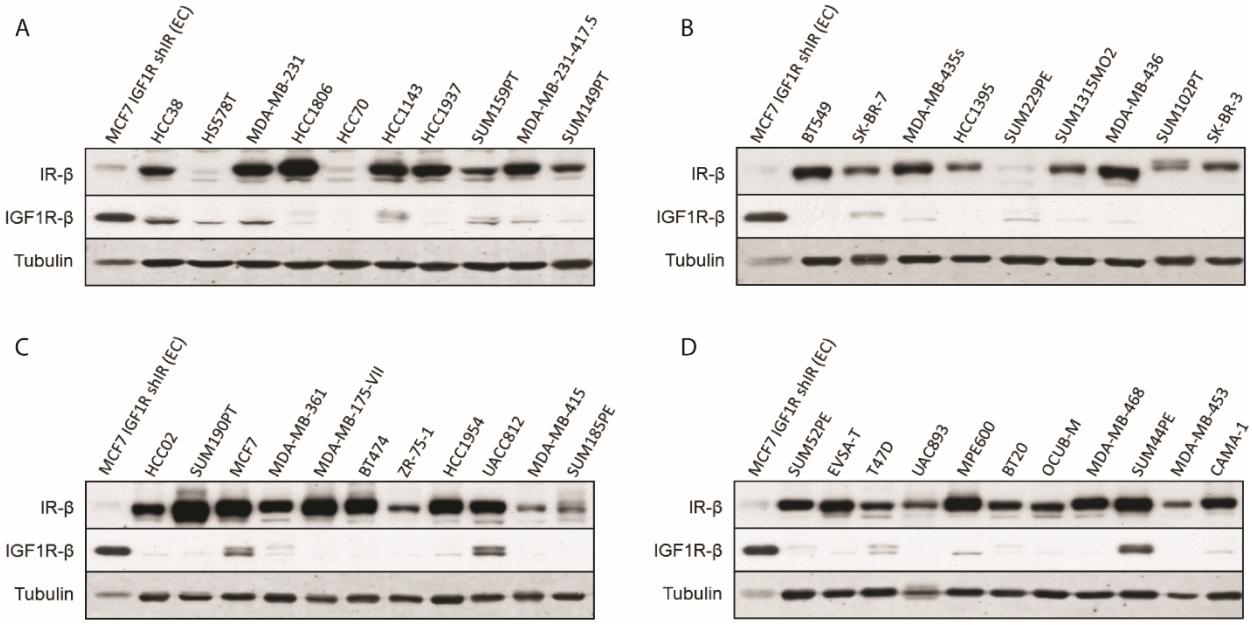
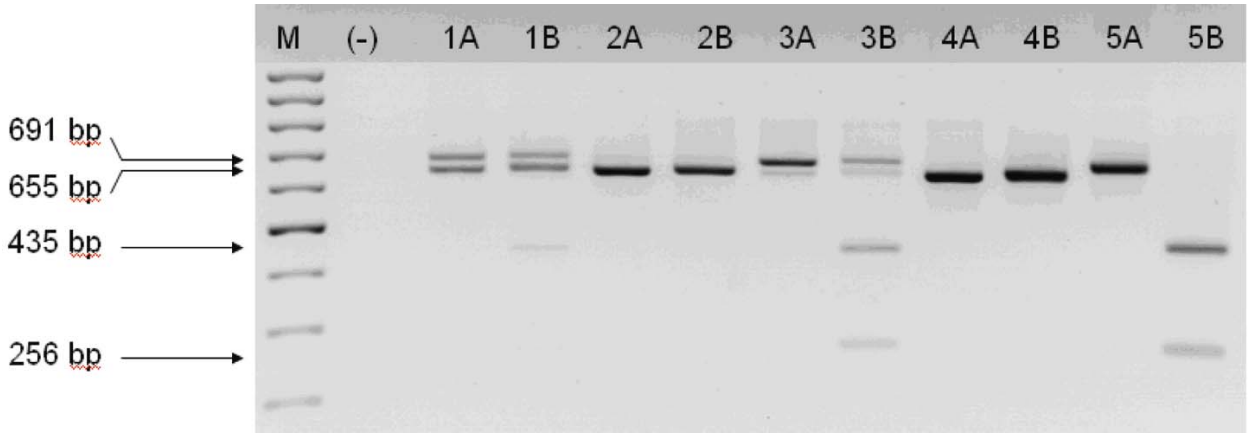
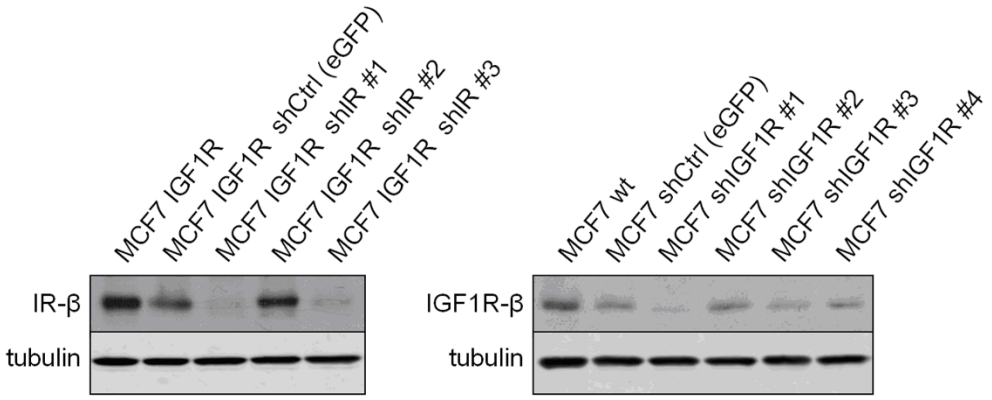
## Supplementary Figures




**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.