PROJECT FINAL REPORT

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Abbreviations

CDK	Chronic Kidney Disease
CI	Confidence Interval
Epo	Erythropoietin
EpoR	Erythropoietin receptor
ESA	EPO Stimulating agents
FFPE	Formalin fixed paraffin embedded
Hct	Hematocrit
HD	Hemodialysis
HR	Hazard Ratio
IHC	Immunohistochemistry
MDS	Myelodysplastic syndromes
MI	Myocardial Infarction
MM	Multiple myeloma
MSM	Marginal Structural Model
PD	Peritoneal dialysis
VTE	Venous thromboembolism
TMA	Tissue microarray

1 Final publishable summary report

1.1 Executive summary

Erythropoietin (Epo), mainly produced by the adult kidney, is the major hormone that promotes erythropoiesis (the production of red blood cells). As such, clinical introduction of the recombinant form of the hormone and its derivatives (Epoetins), has been a breakthrough in treating patients with end-stage kidney disease suffering from anaemia, and cancer patients facing a chemotherapy-associated anaemia. However, despite the vast success in treating anaemia with Epoetins, concerns have been raised regarding the safety of this therapy. The major goal of EpoCan has been to develop and implement a multi-disciplinary strategy to determine Epo associated risks, that include tumour growth progression and thromboembolic events in cancer patients, as well as cardiovascular and cancer risk in chronic kidney disease. EpoCan thus aimed to: (1) Identify, detect and measure possible long-term hazards of Epo treatment; (2) Develop novel prognostic tools; (3) Evaluate the risk-benefit ratio of Epo treatment - to pave the way for new safety and efficacy criteria. To achieve these major aims, EpoCan followed the following steps, considering the fact that Epo acts by binding to its receptor (EpoR) on the target cells: (a) Utilize a wide array of cellular models to thoroughly analyze Epo/EpoR interaction and signaling; (b) Establish and test new, personalized, predictive tools (EpoR peptide antagonists, novel specific anti-EpoR monoclonal antibodies, thromboembolic tests); (c) Create new experimental models of mice to be used as hosts for tumour implants and subjected to Epo; (d) Screen and analyze clinical databases.

EpoCan has developed and validated novel and specific antibodies towards EpoR. These antibodies allowed several profound discoveries, as well as established new reliable tools in the Epo-EpoR field. Additionally, novel animal models genetically modified to form spontaneous tumour formation, coupled with low or high Epo levels, were developed. These models have led to important insights of Epo contribution to tumour growth and metastasis. The major outputs of the EpoCan project are presented below:

- Unravelled that Epo has no direct effect on the function of platelet cells that are involved in thromboembolic events.
- Demonstrated that in patients treated with a high dose of Epo for a short term there were no enhanced proinflammatory or prothromboembolic changes in the circulating blood in cases of acute myocardial infarction (MI). Long term follow-up of the patients showed the safety of this high dose Epo regimen.
- Demonstrated that resistance to Epo stimulating agents (ESA) is associated with increased mortality in both HD (hemodialysis) and PD (peritoneal dialysis) kidney patients.
- Demonstrated that in incident dialysis patients, ESA was not associated with an excess of thromboembolic events. On the contrary, the association between ESA use and ischemic stroke seemed even protective. We found no clear association between ESA use and MI, and no evident dose-response relation.
- Confirmed the hypertensive effect of ESA. It is thus questionable whether the effect of ESA on blood pressure could contribute to an increased cardiovascular risk in these pre-dialysis patients.
- Patients treated with high ESA dose showed a 1.2-1.5 increased risk of mortality. These results support guidelines advising a conservative ESA dosing regimen, which carefully weighs the patients' benefits and risks.
- Elaborated the understanding of EpoR signalling in cancer cells; EpoR homodimerization is taking place in cancerous cells and the formed EpoR dimers are biologically active (i.e. response to Epo stimulation). The data also demonstrate that sufficient levels of the Janus kinase 2 (JAK2) are a prerequisite for EpoR activation.
- Elaborated the understanding of Epo contribution to cancer formation, propagation and metastasis, using novel mouse models with spontaneous tumour formation:
    - Epo overexpression leads to a later onset of tumour formation, suggesting that Epo might be anti-tumorigenic - before a tumour is formed. The deletion of EpoR in spontaneously developing tumours causes an earlier tumour onset, confirming the result that EpoR might act in delaying cancer initiation.
    - Epo overexpression results in faster tumour growth, suggesting that as soon as a tumour has been formed, Epo promotes its growth.
    - Animals bearing tumours that lack EpoR displayed induced tumour growth after Epo administration; suggesting that Epo might promote tumour growth independently of EpoR expression in the cancer cells.
- Elaborated the understanding of Epo contribution to cancer formation, propagation, and metastasis using tumour xenografts models carrying human breast and lung cancer cell lines:
    - Epo stimulates lung cancer cell growth (especially A549).
    - Epo affects the metastatic potential of cancer cells.

In summary, the results of the EpoCan project generated important new knowledge of major adverse events caused by Epo treatment, such as the increased mortality of patients treated with high ESA dose. Nevertheless, it was demonstrated that Epo has no direct effects on platelet function. The results of the EpoCan will be carefully studied by the scientific community, in order to allow preferable use of Epo in patients, for their safer and increased quality of life.

1.2 Summary description of project context and objectives

Erythropoietin (Epo), mainly produced by the adult kidney, is the major hormone that promotes erythropoiesis. As such, clinical introduction of the recombinant form of the hormone (rHuEpo) and its derivatives (Epoetins), has been a breakthrough in treating patients with end-stage kidney disease suffering from anaemia, and cancer patients facing a chemotherapy-associated anaemia[1]. Worldwide annual sales of Epo drugs in 2003 were estimated at $10.3 billion. Despite the vast success in treating anaemia with Epoetins, concerns have been raised regarding the safety of this therapy. EpoCan project was designed to address these safety concerns and identify relevant parameters that will allow better patient selection for Epoetin therapy. The results of this project are expected to yield both improved medical practice, as well as substantial savings to healthcare systems by avoiding severe complications and unnecessary expenses.

Anaemia appears to be an independent poor prognostic factor for survival in patients with cancer. rHuEpo has thus been introduced to alleviate anemia in chemotherapy and radiotherapy treated cancer patients, thus avoiding repeated blood transfusions. Some studies however, have raised concerns that rHuEpo therapy may be harmful to certain groups of cancer patients. In 2003, Henke described the outcome of rHuEpo treatment in a randomized clinical trial of 351 anaemic head and neck cancer patients undergoing radiotherapy[2]. Unexpectedly, loco-regional progression-free **survival was worse in the Epo treatment arm, compared to the placebo group**. In the same year, the Breast Cancer Erythropoietin Survival Trial (BEST) reported poorer **overall survival in patients assigned to the Epo arm**[3]**.** A recent FDA alert has once again highlighted concerns about potential adverse effects of treating cancer related anemia. These publications, coupled to recent unexpected findings that the Epo receptor (Epo-R) is expressed in numerous tumour cell lines and carcinomas, have raised concerns that administration of Epoetins may have a **direct or indirect growth** promoting action on the cancer cells, and thus worsen the prognosis of Epoetin treated cancer patients[4].

On another front, previous studies in end-stage renal disease (ESRD) patients have shown that both high levels of haemoglobin (Hb) and high doses of Epoetin are associated with an increased risk of cardiovascular (CV) and thrombotic events and mortality in these patients[5]. It was maintained that chronic inflammation, often present in patients with severe chronic kidney disease, might play a role in the development of CV and thrombotic events and mortality. The risk of cancer - as related to Epoetin use in these, patients is unknown.

In contrast to these 'red flags' mentioned above, Epo was found by numerous studies to have beneficial **neuroprotective, cardio-protective and immune-mediated anti-cancer effects,** as revealed - for example, in multiple myeloma (MM) subjects[6]. These effects, at least in part, are mediated by EpoRs present on cells other than the erythroid lineage. Hence, in the clinical setup, it is imperative to be aware of, and to consider **risk benefit ratio of Epoetin treatment**. By mere definition of the topic, it is clear that randomized trials to assess the risks of Epoetin treatment in human subjects are not feasible. Therefore, cultured cell lines, mouse models, human tissue samples and data mining are extremely valuable aspects and research strategies regarding the present multifaceted project.

Hence, 25 years after the introduction of rHuEpo into clinical practice, it became essential to re-evaluate the databases of Epo-treated renal patients, as well as to design experimental strategies to determine those cases in which treatment with Epo would be beneficial and avoid those where it may present a hazard. The overarching goal of EpoCan was thus to develop and implement a multi-disciplinary strategy to **determine Epoetin-associated risk of tumour growth progression and thromboembolic events in cancer patients, and cardiovascular and cancer risk in chronic kidney**

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2 Henke, M., Laszig, R., Rube, C., Schafer, U., Haase, K. D., Schilcher, B., Mose, S., Beer, K. T., Burger, U., Dougherty, C. and Frommhold, H. (2003) Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. Lancet. 362, 1255-1260

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[4] Brown, W. M., Maxwell, P., Graham, A. N., Yakkundi, A., Dunlop, E. A., Shi, Z., Johnston, P. G. and Lappin, T. R. (2007) Erythropoietin receptor expression in non-small cell lung carcinoma: a question of antibody specificity. Stem Cells. 25, 718-722

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[6] Mittelman, M., Zeidman, A., Kanter, P., Katz, O., Oster, H., Rund, D. and Neumann, D. (2004) Erythropoietin has an anti-myeloma effect - a hypothesis based on a clinical observation supported by animal studies. Eur-J-Haematol. 72, 155-165

disease. EpoCan aimed to identify those cases where adverse events of Epoetin treatment may be foreseen prior to therapy, via focusing on biomarkers with high sensitivity and specificity. This information is expected to generate the basis for the identification of those patient populations that are at risk upon treatment with the drug. The data accomplished will also form a solid platform for the design of robust conclusive pharmaco-epidemiological clinical trials, with defined measurable endpoints, as well as inclusion-exclusion criteria.

EpoCan has applied cutting-edge cellular and molecular biology and physiological technologies, as well as novel computational approaches to assess long term risks of Epoetin treatment. The multi-disciplinary consortium developed novel cell and animal experimental models, as well as applied human samples derived from a large bio-bank of patient biopsies and advanced in silico analysis of relevant mega databases. The project has studied the adverse events mainly in breast carcinoma, melanoma, ovarian and non-small cell lung carcinoma, as well as in Chronic Kidney Disease (CKD) patients. The output of the project will have far-reaching implications for human health, enhancing the knowledge, and thus optimizing Epoetin treatments. EpoCan derived data will facilitate prediction and prevention of possible long term adverse events such as tumour exacerbation, thromboembolic and cardiovascular side effects, following Epoetin treatment of cancer and chronic kidney disease patients.

The following are the original objectives of EpoCan project – which were achieved in course of the project.

Objective 1. **Determine EpoR expression, signalling cascades and interacting molecules *in vitro* in normal and malignant cells and EpoR expression in human-derived tumour biopsies**

1.1. Generation and validation of EpoR specific antibodies, then detection of EpoR protein in cultured tumour cell lines
1.2 Determine EpoR expression on tissue samples from patients' tumour biopsies of breast carcinoma, melanoma, ovarian and non-small cell lung carcinoma
1.3 Identification of EpoR homodimers and heterodimers active forms, conferring receptor function in normal and in malignant cells
1.4 Analysis of Epo-R downstream signalling components in normal versus malignant cells
1.5 Experimental setup for measuring reduced tumour tissue oxygenation as indication for reduced Epo activity: Imaging HIF-1 (hypoxia inducible factor-1) active form in tumour microspheres.
1.6 Determine the *in vitro* efficacy of antagonistic Epo peptides

Objective 2. Generate new Epo over-expressing mouse models, tailored to determine Epo effect on tumour incidence and growth rate

2.1. Generation of tumour-prone and immunodeficient transgenic mice over-expressing human Epo
2.2. Xenograft transplantation of melanoma, breast cancer, non small cell lung carcinoma and ovarian carcinoma into the human Epo over-expressing models and analysis of tumour growth
2.3. Assessment of Epo-associated tumour risk in (preconditioned) transgenic mouse models

Objective 3. Study EpoR - mediated effects in mouse tumour models

3.1. The role of tumour cell surface expressed EpoR in stimulating tumour growth and metastasis
3.2 Epo-driven tumour growth and/or metastasis via systemic and/or tumour surrounding tissue
3.2.1. Effects of Epo on endothelial cells, pericytes, fibroblasts and myeloid cells types invading tumour mass
3.2.2. Effects of Epo on platelet-tumour cell interaction and its role in tumour dissemination and metastasis
3.3. Tumour hypoxia, in correlation to tumour growth rate and metastasis events, as an indicator of Epo effects.

Objective 4. Determine Epo effects on haemostasis, in particular on platelets, with respect to the risk of thromboembolic events in mouse models and cancer patients

4.1 Analyze blood samples from healthy donors: Epo direct effects on platelet reactivity and function
4.2 Determine the effects of Epo on thrombosis in mouse models
4.3 Study the currently available MEGA data base of venous thrombotic patients to unravel possible correlation between Epoetin treatment and thrombolytic events in cancer patients (relating to type of cancer, treatment protocol, patient age, gender, medical history, etc.)

Objective 5. Assess cardiovascular and cancer risks in CKD related mouse models and in patients' databases

5.1 Develop and exploit new mouse models to study the effect of Epoetin on the progression of atherosclerosis, and thrombosis
5.2 Analyze large data bases related to chronic kidney disease patients, in accordance with the in vivo analysis and with respect to, as follows.
5.2.1. Epoetin associated risk of arterial and venous thrombotic events
5.2.2. Effect of Epoetin on prothrombotic and proinflammatory changes

Objective 6. Results integration and analysis using newly generated computational tools to predict and optimize benefit to risk ratio of Epoetin treatment

6.1 Analysis, evaluation and verification of the collected results, with subsequent optimization of experimental design, using currently available and newly developed advanced bioinformatics and analytical tools.

6.2 Design an integrated data base in silico platform to combine results from all research components: cell lines, mouse models and patients' data bases.

6.3 Data mining analysis to identify and define a rule based on Epo related adverse events to different patients' medical records (e.g. age, gender, type and period of treatment, etc.) for both cancer and CKD patients.

6.4 Translating all accumulated knowledge and data into regulatory schemes optimizing benefit/risk ratio of Epoetin treatment.

6.5 Translating the studies on Epo-R and on Epoetin patient-treatment to a clinical insight.

6.6 Conclusions: exploitation and implementation of project results, towards the generation of regulatory recommendations for safer Epoetin usage.

Objective 7. Dissemination, Exploitation, and Training activities

7.1 Dissemination.

7.2: Training activities

7.3: Exploitation strategy

Objective 8. Management and coordination

8.1 Quality control and risk management

8.2 Administrative and financial coordination

8.3 Intra-consortium communication strategy

1.3 Description of the main S&T results/foregrounds

1.3.1 Epo-R expression, signalling cascades and interacting molecules *in vitro*

Summary of significant results and overall achievements

- Generated and validated specific EpoR antibodies. These antibodies will revolutionize the Epo-EpoR research field and may be useful for patient stratification prior to Epo treatment.
- A total of 27 antibodies generated within EpoCan have been validated for their ability to recognise EpoR by immunohistochemistry (IHC).
- Two rat monoclonal antibodies, BCO-3H2-D3 and BCO-4B5-C9 are valuable for comparative IHC studies in a wide range of cell lines and human tumour tissues.
- BCO-3H2-D3 recognises full length EpoR and BCO-4B5-C9 recognises both full length and truncated EpoR.
- IHC studies have been performed using tissue microarray (TMA) samples from 519 cancer patients with lung, breast and ovarian oesophageal cancer.
- Patient IHC results were collated with their clinical data by the Pathology Integromics in Cancer system which combines data from several sources, including patient databases, digital pathology archives and TMA results.
- Confirmed Epo-R homodimerization in living non-erythroid cells, demonstrating the effect of Epo on other organs aside from the blood system.
- Successfully used STAT5 redistribution analysis to assess EpoR activity in living cells.
- Discovered a role for JAK2 as potential bottleneck that may impede proper EpoR signalling when not sufficiently expressed.
- Successfully used pO2 sensitive nanoparticle probe to assess cellular oxygenation levels.
- Data showing that Epo-R homodimerization is principally taking place in cancerous cells, and that the formed EpoR dimers are biologically active (i.e. respond to Epo stimulation). The data also show that sufficient JAK2 levels are a prerequisite for receptor activation. This may indicate that although certain tumour lines express EpoR, functionality may be reduced, depending on the expression of other molecules such as JAK2.

Generation and validation of Epo-R specific antibodies, detection of EpoR protein in cultured tumour cell lines

Antibodies towards denatured EpoR are a valuable tool for biochemical studies and for the clinical research of Epo effects and involvement in disease. In order to generate antibodies required for analysis of denatured human EpoR, a mixture of 6 EpoR-specific peptides derived from various regions of the EpoR protein were used to immunise rats in order to generate monoclonal antibodies. Rigorous testing identified one monoclonal antibody (GM1201) that passed all specificity tests, recognising a peptide from the cytoplasmic region of EpoR. For antibodies required to recognise the native human EpoR protein, genetic immunisation using the total extracellular coding region was applied to rats and mice. Rigorous testing identified three monoclonal antibodies that passed the specificity tests (GM1202, GM1203 and GM1204).
For the immunoprecipitation studies, the native human EpoR protein was precipitated from cell lysates, followed by denaturation and analysis on Western blots in different combinations. These studies further confirmed the specificity of the different antibodies. Specificity was further confirmed by reducing the EpoR protein levels using siRNAs and a final proof identified several EpoR peptides following immunoprecipitation, proteinase digestion and mass spectroscopy. The generated antibodies are essential and valuable tools for future studies in the Epo field.
Widespread availability of these antibodies should enable the research community to gain a better understanding of the role of EpoR in cancer, and eventually to distinguish patients who can be treated safely by rHuEpo - from those that are at increased risk from the treatment.

EpoR expression on tissue samples from patients' tumour biopsies of breast carcinoma, melanoma, ovarian and non-small cell lung carcinoma

A group of 27 antibodies that were developed within EpoCan were validated for their efficiency in recognising EpoR by immunohistochemistry (IHC). Initially the antibodies were tested for their ability to bind to UT-7 cells, an erythroid cell line with high EpoR expression, and differential binding to two B cell lines: REH cells with high EpoR expression and NALM cells with low EpoR expression. The 24 monoclonal antibodies were then screened in IHC studies on archival clinical samples usually from formaldehyde-fixed paraffin-embedded (FFPE) blocks. The two rat monoclonal antibodies, BCO-3H2-D3 and BCO-4B5-C9, proved to be valuable in comparative IHC studies in a wide range of human tumours and cell lines. Using immunoprecipitation (IP) and Western blot (WB) studies it has been deduced that BCO-3H2-D3 recognises full length EpoR and that BCO-4B5-C9 recognises both the full length and truncated forms of the receptor.

To date IHC studies were performed of FFPE lung tumours (10 cases), breast tumours (290 cases) and ovarian tumours (219 cases) on tissue microarray (TMA) samples, in conjunction with the Northern Ireland Biobank using BCO-3H2-D3 monoclonal antibody. To integrate the IHC results with the well-documented available clinical data, the QUB group has utilised in silico databases such as the one maintained by the Northern Ireland Biobank (NIB). Data were collated by the Pathology Integromics in Cancer (PICan) system which combines results from several sources, including patient databases, digital pathology archives and TMA results. The two rat monoclonal antibodies, BCO-3H2-D3 and BCO-4B5-C9, should enable careful dissection of EpoR function in tumour tissue, help to clarify the possible role of EpoR isoforms in tumourigenesis, and lead to further publications from the EpoCan partners.

Identification of EpoR homodimers and heterodimers active forms, conferring receptor function in normal and in malignant cells

Plasmids encoding for full-length human EpoR labelled with enhanced cyan and yellow fluorescent protein (CFP and YFP, respectively) were generated and inserted into different cells in order to test the mechanism the EpoR activation and signalling. Although both constructs show excellent co-localization in confocal fluorescence microscopy, it was not possible to confirm dimer formation by Fluorescence Resonance Energy Transfer (FRET) microscopy.

A modified ECFP/EYFP labelled EpoR construct, where the last 203 cytoplasmic amino acids were deleted, showed dramatically increased localization to the cytoplasm membrane. Between these truncated chimeras FRET, signals that are significantly above background levels were detectable.

These data are evidence for the formation of EpoR dimers inside the observed cells.

Interestingly, FRET was not only detectable at the cytoplasm membrane but also in the endoplasmic reticulum and in the Golgi compartment, suggesting that dimer formation is taking place at an early stage of EpoR synthesis.

In time lapse experiments, no change of FRET efficiency upon ligand addition was detectable. **We conclude that the described truncated EpoR dimer does not change its conformation upon ligand binding.**

When the fluorophore was cloned between JAK2 binding site and the remaining tyrosine residues, we obtained a construct that was as signalling competent as the wild type receptor. However, we were not able to measure significant FRET signal with this type of construct. We assume that (despite successful dimerization) ECFP and EYFP are too far separated (or oriented) - to allow for detectable FRET.

Analysis of EpoR downstream signalling components in normal versus malignant cells

Epo-R signalling requires intracellular molecules for activation of a tyrosine phosphorylation dependent signalling cascade ultimately leading to activate transcription factors such as STAT5 that enters the nucleus to induce gene expression in cells responding to Epo treatment.

Of importance, the requirement of sufficient amounts of these intracellular signalling components has not been systematically studied with respect to EpoR activation. Association of EpoR with the JAK2 is sufficiently documented and was confirmed, and transfection with JAK2 increased the membrane localisation of EpoR.

We then successfully used a STAT5-EYFP fusion construct to assess Epo-R activation in living cells. In EpoR positive cells, a redistribution of STAT5 to the nucleus was observed within 10 minutes after ligand addition. EpoR negative cells did not exhibit any changes in STAT5 distribution upon stimulation with Epo. Interestingly, we noticed that the described redistribution is by far more likely to happen, when JAK2 is also overexpressed.

This result demonstrates the role of JAK2 as a bottleneck in the signalling cascade that may impede proper EpoR signalling in non-erythroid cells.

Experimental setup for measuring reduced tumour tissue oxygenation as indication for Epo activity: Imaging HIF-1 active form in tumour microspheres

In addition to its erythropoietic activity, Epo has been claimed to induce angiogenesis, in particular, in solid tumours. As a consequence, tumour oxygenation will improve and will thus lead to changes in the transcriptional signature of tumour cells. One central transcription factor driving hypoxia-induced gene expression in tumour cells is HIF-1. As FRET signal between the fusion constructs was found to be independent of pO_2 (observation in monolayer cells), another way to measure tissue oxygenation had to be found. An oxygen-sensitive, multispectral, multimodal dye was therefore successfully tested.

The dye can successfully be excited by multi-photon (here: two-photon) excitation microscopy facilitating imaging also deep within three-dimensional tumours/spheroids.

The experiments demonstrated that oxygen quenches the phosphorescent signal reproducibly. The dye exhibits a multispectral profile: by normalizing the oxygen dependent part (around 660 nm) to the oxygen insensitive part (around 440 nm) the measurement can be rendered independent of concentration changes.

A method was established for generation of tumour microspheres that is also working sufficiently well. Like tumours not being perfectly spherical, the generated multicellular objects have clearly a 3-dimensional and compact morphology, making them well-suited for tests with the oxygen sensitive dye inside these spheroids. Initial tests with Hoechst 33342 stained spheroids led to strong signals. As the MM2 probe and Hoechst 33342 have quite similar excitation characteristics (both are excitable in the near UV range in single photon mode), good signals are to be expected from experiments with the MM2 dye inside multicellular objects.

Determine the *in vitro* efficacy of antagonistic Epo peptides

With the aim of generating Epo specific antagonists, to locally reduce Epo action on cells other than the erythroid lineage, EpoCan has found that Epo derived synthetic peptides can act as Epo-R antagonists *in vitro* on UT7 cells and on Ba/F3 cells expressing Epo-R. Hence, upon further exploitation for selective targeting of the peptides, these Epo antagonists may prove useful to enable Epo effects on erythropoiesis, while avoiding the potential reported risks.

1.3.2 Generate Epo mouse models, tailored to determine Epo effect on tumour incidence and growth rate

Summary of significant results and major achievements

1. Generation of mouse models with spontaneous tumour formation that over-express Epo or that received Epo injections mimicking patient situations. We established mice with tissue (mamma and skin) specific Trp53 deletion that develop tumours, and compared Epo overexpressing with control, as well as Epo injected compared to control mice. In addition, we introduced another model that forms spontaneous mamma tumours that lack EpoR, on genomic level ($EpoR^{flox}$). The following results were obtained:

- Epo overexpression leads to a later onset of tumour formation, suggesting that Epo might be anti-tumorigenic before a tumour cell generates.
- The deletion of EpoR in spontaneously developing tumours causes an earlier tumour onset, confirming the result that EpoR might delay cancer initiation.
- Epo overexpression results in faster tumour growth, suggesting that Epo promotes tumour growth, as soon as a tumour has been formed.
- Epo injection did not lead to a change in survival of tumour-bearing animals, suggesting that very high levels of Epo might be needed to promote tumour growth.
- Animals with tumours lacking EpoR displayed induced tumour growth after Epo administration, suggesting that Epo might promote tumour growth independently of EpoR expression in cancer cells.

2. Analysis of tumour xenografts models carrying human breast and lung cancer cell lines: We used nude mice and injected them with Epo. The following results were obtained:

1. Epo stimulates cancer cell growth in lung cancer cells (especially A549)
2. Epo affects metastatic potential of cancer cells
3. The loss of EpoR is associated with reduced tumour cell growth *in vitro* (MDA-MB-231 and A549) and also *in vivo* (A549), and suggests that EpoR plays an essential role – at least in the cell lines tested, and this effect is independent of Epo injections

Our experiments raised new questions; especially one experiment suggests that EpoR might not need to be expressed in tumour cells to confer an Epo effect. Future studies should explore if Epo is indeed able to affect tumour growth and metastasis - independently of the EpoR status in the cancer cells.

Generation of tumour-prone, atherosclerotic and immunodeficient transgenic mice over-expressing human Epo

Tumour-prone mouse models

The original plan in the grant application was to combine Tg(Epo) [high levels of Epo] with a constitutive knockout mutation of the tumour suppressor gene, Trp53. Because this animal model is highly tumour prone and spontaneously develops a spectrum of different tumours (about 75% are lymphomas), we decided to extend our research program. To improve the predictability and controllability of tumours we combined Tg(Epo) [high levels of Epo] with conditional and tissue specific Trp53 knockout mutations. Additionally, we generated mice with tumour specific deletion of the EpoR. Altogether, we developed five novel double and triple mutant lines by crossbreeding.

The novel mouse models were genetically and phenotypically characterized. By characterizing the newly developed strains we found out that the average life expectancy of mice with Tg(Epo) [high levels of Epo] background was shorter than the tumour latency (17.6 vs. 21.7 weeks) probably due to the high haematocrit induced by Epo overexpression. Therefore, we decided to splenectomise all animals, including a control group, in order to reduce the haematocrit and to increase the life span of the animals. After splenectomy, the life span of the triple mutants was comparable to that of the parental Trp53 knockout mice, although the haematocrit was only slightly reduced from the pre-splenectomised Tg(Epo) mice. Therefore, this animal model is useful to determine whether Epo treatment presents a risk for patients with cancer. This investigation is still going on, because we need to wait until all animals of the ten experimental groups have developed the expected tumours.

Xenograft transplantation of melanoma, breast cancer, non small cell lung carcinoma and ovarian carcinoma into the human Epo over-expressing models and analysis of tumour growth

As an alternative approach to determine whether Epo treatment presents a risk for patients with cancer, we conducted xenotransplantation experiments using immunodeficient nude mice as recipients for human breast cancer cells. The cancer cells were genetically modified to express luciferase as a marker for tracing tumour development and metastasis after orthotopic transplantation. Furthermore, the cells were genetically modified to induce a conditional knock down of the EpoR (linked to red fluorescence protein expression) *via* Dox treatment. Tumour recipient mice were treated with recombinant human Epo, with or without down regulation of the Epo-receptor in the tumour cells.

All 20 animals developed the expected mammary tumour at the site of inoculation. Metastases could be identified by *in vivo* imaging, using the luciferase reporter. Metastases were found in lymph nodes, lungs, and spleen and were confirmed by histology of the organs. We found no significant differences in tumour growth among the treatment groups.

However, downregulation of the EpoR appears to support metastasis in the transplanted animals. This finding has to be further investigated.

In addition to breast cancer cells, we analysed human lung cancer cells (A549) in nude mice. Importantly, EpoR knock down leads to a growths disadvantage *in vitro*. Therefore, several knock down and control clones were generated in order to maintain stable clones with a persistent EpoR ablation. We implanted A549 clones subcutaneously into immunodeficient Foxn1nu mice. Whereas EpoR knock down clones showed no difference between Epo and NaCl administration, genetically unmodified A549 cells displayed significantly induced tumour growth in Epo treated animals. Interestingly, EpoR knock down clones were growing at a slower rate (as observed *in vitro*, and independent of Epo administration) than their respective control clones, suggesting that EpoR might utilize to a large extend Epo that is already endogenous. Analysing metastasis is still on-going, but our preliminary data suggest that Epo boosts metastasis of both, EpoR knock down and wild type lung cancer cells.

Assessment of Epo-associated tumour risk in (preconditioned) transgenic mouse models

We analysed the models of spontaneous tumour formation, in order to test the impact of administrated or overexpressed Epo (Tg6 background) on tumour incidence, proliferation and metastasis. The Following models were analysed:

1. WapCre;Trp53$^{flox/flox}$

WapCre; Trp53$^{flox/flox}$ mice develop mamma specific tumours between 18 and 28 weeks of age. We treated mice with Epo or NaCl solution (control) after the tumour was diagnosed (by palpitation).

We observed that Epo injections did not reduce the animal survival time (time until the animal is reaching the termination criteria of the experiment), and there was no significant difference in metastasis formation between the Epo and the control groups. However, it has to be mentioned that some of these mice develop a tumour-associated anaemia that could not be restored with Epo administration (Epo activity was confirmed in tumour free animals) and that might interfere with our analyses of the results.

2. WapCre;Trp53$^{flox/flox}$;Tg6(Epo)
These mice develop like the previous model of mamma specific tumours, due to the deletion of p53. In addition, these mice overexpress Epo (Tg6 background) and have therefore chronically elevated levels of Epo.
We found that these mice have a later tumour onset - compared to their sibling controls (without Tg6 Epo overexpression), and a faster tumour growth; suggesting that **Epo might protect from tumorigenic events in the first place, but promotes tumour growth as soon as a tumour has developed.**

3. WapCre;Trp53$^{flox/flox}$;EpoR$^{flox/flox}$
As a counterpart to the previous Epo overexpressing model (Tg6) we generated mice with a mammary tumour specific deletion of EpoR in order to analyse the impact of Epo-EpoR on tumour growth. In accordance with our findings above (Tg6 Epo overexpression delays tumour generation) we observed that mice with a mamma specific deletion of EpoR (WapCre; Trp53$^{flox/flox}$; EpoR$^{flox/flox}$) developed tumours earlier (30 – 40 weeks of age) than their respective sibling controls (WapCre; Trp53$^{flox/flox}$; EpoR$^{flox/wt}$) (35-55 weeks of age). We treated both groups with Epo, or with NaCl solution, and observed that mice with EpoR ablation (WapCre; Trp53$^{flox/flox}$; EpoR$^{flox/flox}$) displayed reduced survival (time till reaching experimental termination criteria), suggesting that Epo might stimulate tumour growth independently of EpoR expression in tumour cells.

1.3.3 Study EpoR - mediated effects in mouse tumour models

Summary of significant results and overall achievements

In order to investigate the relative contribution of EpoR expressed in tumour cells versus that in host cells on the Epoietin-driven protumoral activity, it is essential to study tumour cells that lack EpoR expression or silencing its expression in EpoR-expressing tumour cells.

As models of tumour cells that express the EpoR we employed melanoma (B16-F10), leukemia (UT7), breast carcinoma (MDA-MB231) and non small cell lung carcinoma (A549). As a model for a tumour that does not express the EpoR we used Lewis lung carcinoma (LLC). This latter model was supposed to enable measuring Epo-induced changes in tumour progression that are due to EpoR signalling, in cells that are derived from the host and not from the tumour.

However, we first found that **EpoR is broadly expressed in tumour cells including LLC**, originally planned as EpoR-deficient cell line relevant to assess the role of tumour stroma in Epo-driven protumoral activities. Therefore, since we have not found any single natural occurring EpoR negative tumour cells, the relative contribution of tumour cells - versus tumour stroma cells, on EpoR-driven protumoral activities, cannot be assessed in LLC cells and should be necessarily addressed in EpoR-silenced cells.

Nevertheless, **silencing of EpoR in tumour cells reduces its competitiveness when compared with EpoR-expressing control cells,** and as a consequence, the EpoR silencing efficacy is progressively lost. These data point to the cell autonomous importance of EpoR in tumour cells. Therefore, the generation of EpoR silencing cells to assess their xenograft formation capability requires the selection of EpoR silenced clones, to avoid progressive lost of EpoR silencing.

We isolated clones from EpoR-silenced A549 cells that show a good decline of EpoR protein. These clones in which EpoR is permanently declined are **suitable to growth of xenografts** in different mouse models and then to assess the relationship between Epo levels, EpoR presence or absence on the tumour, Hb values and disease outcome. The new generated tools of EpoR silenced cells will thus be valuable tools for research on the effect of Epo on cancer growth that is not mediated via the EpoR.

The role of tumour cell surface expressed Epo-R in stimulating tumour growth and metastasis.

The use of Epo has been challenged due to the notion that they might enhance tumour initiation and propagation. However, it remains completely unknown whether this pro-tumoural effect is mediated by binding to its receptor (EpoR) present in tumour cells, or EpoR present in host cells invading the tumour mass (tumour host stroma). In order to

investigate the relative contribution of EpoR expression in tumour cells versus host cells in the Epo-driven protumoural activity, it is completely essential to study tumour cells that lack EpoR expression, or silencing its expression in EpoR positive tumour cells. As a model for a tumour that does not express the EpoR, we included in the original proposal the LLC (Lewis lung carcinoma) cell line, because these tumour cells have undetectable levels of EpoR. Therefore, this cell line was considered proper for studying the effect of EpoR on tumour growth, and was really helpful to assess the relative contribution of tumour stroma on the protumoural effects of EPO.

In this regard, EpoR protein has been measured by Western Blot in different human and mouse tumour cell lines, such as UT7 (leukemia), A549 (human lung carcinoma), MDA-MB-231 (human breast carcinoma), B16-F10 (murine melanoma cell line) as well as LLC. We found that EpoR protein expression signal is very high in UT7 and more modest (but clearly detected) in MDA-MB-231, A549 human cells. It is important to mention that EpoR protein signal was also detected in mouse B16-F10 and LLC cells.

Therefore, **LLC cells (i) cannot be considered as representing EpoR negative cells, and (ii) cannot be used to investigate whether Epo can promote tumour growth independently of its expression in tumour cells, as originally proposed.** Therefore, it was necessary to use EpoR-silenced tumour cells to understand the role of tumour cell versus tumour stroma, because we have not found any single naturally occurring EpoR negative tumour cells.

We planned to use EpoR-silenced A549 human cell lines, in which we have successfully silenced their endogenous EpoR expression, to generate EpoR-silenced xenografts. However, during the preparation of EpoR-silenced cells we observed that the remarkable silencing of EpoR in A549 was progressively lost over time, and the silencing was minimal after a few weeks in culture.

Silencing of EpoR in tumour cells reduces its competitiveness, and as a consequence, the EpoR silencing efficacy is progressively lost. These data indicate the importance of EpoR expression in the biology of tumour cell competitiveness, which in some extent can provide molecular/cellular insight into the protumoural of effects of EPO. Therefore, the generation of EpoR silencing cells to assess their xenograft formation capability requires the selection of EpoR silenced clones, to avoid progressive lost of EpoR silencing. We have thus succeeded to isolate 5 clones from shSCR A549 cells and 20 from shEpoR A549 cells. From the former we have selected four clones and from the latter - three clones (the three that express less EpoR protein amount). ***These clones were used for injection in nude mice to investigate the relative contribution of EpoR expression in tumour cells versus host cells in the Epoetin-driven protumoral activity.***

Tumour hypoxia, in correlation to tumour growth rate and metastasis events, as an indicator to Epo effects

Hypoxia is a shortage of oxygen which is observed in many tumours when they grow beyond a certain size and oxygen demand can no longer be matched by supplying blood vessels. Hypoxia has been recognized as an independent risk factor in malignant disease, and in general it involves the activation of hypoxia inducible factor 1 (HIF-1) which is under the control of NFκB. Impinging on this signaling cascade with EpoR activation may thus affect oxygen homeostasis in tumour cells and tissues. On the other hand, changes in haematocrit upon Epo treatment will also affect oxygen supply and thus tissue oxygenation.

Intra-tumour hypoxia is a sign of insufficient tumour perfusion closely related to the inefficiency of intra-tumour vascular network. Intra-tumour vascular dysfunction leads to an insufficient supply of blood and oxygen to the inner core of the tumour, aggravating tumour hypoxia. The appearance of intra-tumour hypoxia can potentiate the intrinsic migratory potential of tumour cells. Therefore, vessel leakage not only favours tumour metastasis enabling tumour cells to escape from the primary tumour, but can eventually cause a hypoxia-induced tumour cell migration response in poorly oxygenated tumour areas. Overall intra-tumour hypoxia is a risk for tumour metastasis and dissemination.

Because we found EpoR is broadly expressed in tumour cells, including LLC (originally planned as $EpoR^{negative}$ cell line), we wondered whether its levels could be modified in hypoxic conditions. In this regard, A549, MDA-MB-231, LLC and B16-F10 cell lines were exposed to hypoxia and mRNA levels were analysed by quantitative PCR. We found that EpoR

mRNA levels were significantly higher under hypoxic conditions in A549 cells, compared to normoxia; around two and a half fold. However, no significant differences were found in MDA-MB-231, LLC and B16-F10 cell lines when we compared EpoR mRNA levels in these cells under hypoxic conditions, to those in normoxia.

These results indicate that hypoxia can have different effects on the EpoR expression, depending on the cell line model. Therefore, in order to understand if Epo alters intra-tumour oxygenation in the tumour models having an effect on tumor growth and if this effect is executed through the EpoR expressed by either tumour cells or stroma cells, we need to use EpoR-silenced tumour cells versus control tumour cells to growth xenografts *in vivo* in mice injected with or without Epo.

1.3.4 Epo effects on haemostasis, on platelets, with respect to thromboembolic events risk

Summary of significant results and major achievements

Epo has no direct effects on platelet function. Furthermore, our data show that Epo does not enhance platelet function *in vivo*, indicating that Epo does not affect platelet function via the megakaryocytes. If anything at all, our mouse models show a decreased platelet function. In human patients, Epoetin treatment triggered a weak increase in platelet numbers, but had no effect on platelet function.

Analyse blood samples from healthy donors: Epo direct effects on platelet reactivity and function

We studied the effects of Epo on platelet function in the blood. Platelet activation was measured by P-selectin upregulation and fibrinogen binding, at baseline and at 2, 4, 8, 16, 32 and 64 minutes after activation with agonists.
Using flow cytometry, we measured whether treatment of whole blood with Epo affected the upregulation of P-selectin or the binding of fibrinogen after platelet activation.
We observed no difference in agonist induced P-selectin expression in Epo-treated blood samples as compared with controls.
Effects of Epo on platelet thrombus formation under flow, was studied in whole blood. Real-time video microscopy was used to study whether Epo treatment affected platelet aggregation during perfusion of whole blood over a collagen coated surface. We found no differences in platelet aggregation between Epo treated blood as compared with untreated blood. Using video quantification software, we confirmed that incubation of whole blood with Epoetin did not affect platelet aggregation under flowing conditions as compared with controls.

Determine the effects of Epo on thrombosis in mouse models

Platelet activation in VhL -/- Erythropoeitin overexpressing mice

Using flow cytometry, we examined whether induced overexpression of Epo affected the upregulation of P-selectin after platelet activation with ADP or CRP-XL as compared with wild type mice. We observed a significant decrease in ADP induced P-selectin expression in VhL -/- mice as compared with controls.

Platelet activation in mice overexpressing Epo

Using flow cytometry, we examined whether induced overexpression of Epo affected the upregulation of P-selectin after platelet activation with ADP or CRP-XL, as compared with wild type mice. We found a significant decrease in CRP or PAR4-induced P-selectin expression in Tg6 mice, as compared to the controls.

Study the currently available MEGA data base of venous thrombotic patients to unravel possible correlation between Epoetin treatment and thrombolytic events in cancer patients (relating to type of cancer, treatment protocol, patient age, gender, medical history, etc.)

The effect of Epo on platelet activation and coagulation was studied in patients with acute myocardial infarction (AMI). Five days of treatment with Epo resulted in elevated platelet numbers, as compared to the placebo group. After PCI, platelet aggregation decreased significantly, as expected in response to peri interventional therapy with clopidogrel and aspirin. This effect was most distinctive after 12 and 48 hours respectively. However, there was no significant difference between the Epo and the placebo group. After 60 and 120 hours, platelet aggregation increased gradually but did not yet reach the initial levels, except for TRAP-stimulated platelets.

Several clinical trials in cancer patients have suggested a shorter survival and more venous thrombosis with ESA treatment. The MEGA study is a population-based case control study, designed to define risk factors for venous thrombosis. Among the venous thrombosis patients 696 have been diagnosed with cancer and in the control population 236 patients. Despite considerable numbers of subjects in this study that have been diagnosed with cancer (>900 patients), registered use of Epoetin was low. Only 26 patients within the MEGA database reported the use of Epoetin, 24 in the venous thrombosis patients and 2 in the controls. In view of these low numbers, it was concluded that the MEGA database is not suitable to unravel potential associations between Epoetin treatment and thrombolytic events in cancer patients. Therefore, other possibilities were explored to answer these questions. An excellent opportunity was found in the Danish National Registry of Patients. ESA treatment is currently limited to patients with palliative chemotherapy, except for some patients with myelodysplastic syndromes (MDS) and lymphoproliferative disorders, such as multiple myeloma (MM). Therefore, we will assess in this database the risk of thrombotic events (venous thrombosis, myocardial infarction and stroke) with ESA use in patients with MM and MDS.

1.3.5: Assess cardiovascular and cancer risks in CKD related mice models and in patients' databases

Summary of significant results and overall achievements

- Mouse models to investigate the effect of Epo on thrombosis and atherosclerosis have been developed. Thrombosis and atherosclerosis were enhanced in mice overexpressing Epo. Since Epo also increased haematocrit (Hct), direct and indirect effects of Epo cannot be differentiated using these models. Thus, increased Hct induced by Epo enhances thrombosis and atherosclerosis in mouse models. Yet, the direct effects of Epo remain to be elucidated.
- In patients receiving high dose, short term Epo treatment in acute myocardial infarction, we observed no enhanced proinflammatory or prothrombotic changes in the circulating blood, and long term results showed the safety of this regimen of high dose Epo. This suggests that high-dose for a short term Epo is safe.
- In dialysis patients receiving Epo, platelet activation was decreased - as compared to patients not receiving Epo.
- Epo stimulating agents (ESA) resistance is associated with increased mortality in both HD (hemodialysis) and PD (peripheral dialysis) patients. The effect of ESA resistance, ESA dose and haemoglobin (Hb) are closely related, and the exact mechanism remains unclear. Our results strengthen the need to investigate and relate to causes of ESA resistance not only in HD, but also in PD patients.
- In incident dialysis patients, ESA was not associated with an excess of thrombotic events. On the contrary, we found that the association between ESA use and ischemic stroke seemed even protective. We found no clear association between ESA use and myocardial infract (MI) and no evident dose-response relation.
- We confirmed the hypertensive effect of ESA, since ESA treated patients received more antihypertensive agents. However, no relevant difference in blood pressure (BP) was found between patients with and those without ESA; thus the increase in BP (blood pressure) seems to be controlled for by antihypertensive medication. It seems therefore questionable that the effect of ESA on blood pressure could contribute to an increased cardiovascular risk associated with ESA use in these pre-dialysis patients.
- Patients treated with high ESA dose showed a 1.2-1.5 increased risk of mortality. These results support guidelines advising a conservative ESA dosing regimen, that carefully weighs the patients' benefits and risks.

Develop and exploit new mouse models to study the effect of Epoetin on the progression of atherosclerosis, and thrombosis

ApoE(-/-) and tg(Epo)+/Tg mice were crossed to obtain mice heterozygous at the ApoE locus and transgenic for Epo. These double mutants were crossed again with ApoE(-/-) mice to obtain ApoE(-/-), Tg(Epo)+/Tg mice. This new mouse model is suitable for studying the effect of Epo on atherosclerosis and cardiovascular risks. Mice were fed on a Western diet for 4 weeks, and atherosclerotic lesions of the aorta were analysed macroscopically. We found in ApoE(-/-), Tg(Epo)+/Tg mice as compared to ApoE(-/-) mice formation of atherosclerotic aneurysms and increased atherosclerotic lesions. However, in these ApoE(-/-), Tg(Epo)+/Tg mice the haematocrit was elevated up to 0.8 l/l. Thus, it cannot be excluded that these effects on atherosclerosis are not a direct effect of Epo but due to the increased Hct with enhanced intravascular volume and increased viscosity.

The Tg(Epo) mouse overexpress human Epo in an **oxygen-independent**, manner that simulates the Epo levels of patients treated with Epo. To assess the effect of EPO overexpression on venous and arterial thrombosis, thrombus formation was analysed in mice overexpressing Epo (Tg6 mice) and compared to wildtype mice. In a model of low flow and in an arterial injury model of the carotid artery we found an increased thrombus formation in Tg6 mice compared to wildtype mice. It has to be considered that the mice overexpressing Epo showed elevated Hct, even though splenectomy had been performed on them to reduce Hct levels. Thus, using this model it is not possible to distinguish between direct or indirect effects of Epo on thrombus formation. Mice overexpressing Epo showed two-fold increased urea values as compared to wildtype mice, indicating renal insufficiency.

Analyze large data bases related to chronic kidney disease patients, in accordance with the *in vivo* analysis and with respect to: (1) Epoetin associated risk of arterial and venous thrombotic events and (2) Effect of Epoetin on prothrombotic and proinflammatory changes

Epo associated risk of arterial and venous thrombotic events

The association between ESA resistance and mortality was studied in a large cohort of incident dialysis patients (NECOSAD). ESA resistance was defined as Hb level < 11 g/dL with an above median ESA dose (i.e. 8,000 units/week in HD and 4,000 units/week in PD patients). Unadjusted and adjusted Cox regression analysis for all-cause 5-year mortality was performed for HD and PD patients separately. ESA resistant HD patients had a 37% increased risk of mortality (hazard ratio of 1.37 (95% CI 1.04-1.80) and in ESA resistant PD patients even a more than two-fold increased risk was observed, as compared to patients with a good response, (HR=2.41 (1.27-4.57). These results strengthen the need to investigate and treat causes of ESA resistance not only in HD, but also in PD patients. A report on these results was published *(Suttorp et al., Erythropoiesis-stimulating agent resistance and mortality in hemodialysis and peritoneal dialysis patients. BMC Nephrol 2013;14(1):200)*

The association between ESA and blood pressure was assessed in pre-dialysis patients enrolled in the PREPARE-2 study. This cohort included 502 incident pre-dialysis patients, who started specialized pre-dialysis care in 25 clinics in the Netherlands. When referred to pre-dialysis care, 40.8% of the patients were treated with ESA. Just before start of dialysis, this percentage increased to 58.3%. Overall use of antihypertensive medication was higher in ESA users (95.6% as opposed to 73.1% in non-users at start of pre-dialysis care). Mainly angiotensin receptor blockers and loop diuretics were prescribed more often. No differences in mean blood pressure values were observed between patients with and those without ESA therapy. However, patients treated with a high dose (i.e. > 6,000 units/week) had a 3.7 mmHg (95% CI:-1.6;9.0) higher systolic blood pressure than patients treated with the lowest dose category (≤ 2,000 units/week). Our results support the hypothesis that the use of ESA affects blood pressure. However, it is unlikely that this effect solely explains the increased cardiovascular risk associated with Epoetin use in pre-dialysis patients, as the increase in blood pressure seems to be well controlled for by antihypertensive medication. This study has recently been published *(Suttorp, et al., Effect of erythropoiesis-stimulating agents on blood pressure in pre-dialysis patients. PLoS One. **8**, e84848, 2013)*

We performed additional chart reviews in 6 dialysis centres that participated in NECOSAD. Data on arterial and venous thrombosis events were collected for 805 dialysis patients. During chronic dialysis 58 ischemic strokes, 110 myocardial infarctions and 13 venous thrombosis events were recorded. Patients with ESA had a two-fold lower ischemic stroke rate than patients without ESA (HR adjusted 0.45 (95% CI 0.32-0.90) and a 1.12 (95% CI 0.58-2.14) higher risk of MI. No profound ESA dose response effect was shown. Unadjusted HR for VT was 0.41 (95% CI 0.11-1.50) for ESA treated patients compared to patients without ESA, but the low event rate prevented us from further adjustments. Although in general, the results of observational studies and anaemia-correction trials suggest a harmful effect of ESA on mortality and composite cardiovascular endpoints, it does not seem caused just by an excess of MI or ischemic strokes. These results were recently published *(Suttorp et al., Erythropoiesis-stimulating agents and thrombotic events in dialysis patients. Thromb Res, 2014).*

The association between ESA dose and mortality was assessed in the NECOSAD cohort. Mortality in patients with a high ESA dose (above median 6000 units/week) was compared to patients with a low ESA dose. To handle time-dependent confounding, a sequential Cox approach was used conditional on baseline covariates, with inverse probability of censoring weights (IPCW) for any dependent censoring, including ESA treatment switch and kidney transplant. Analyses

were repeated with a Marginal Structural Model (MSM) with inverse probability weights for ESA treatment and IPCW for ESA treatment switch and kidney transplant. Weights were stabilized and based on age, gender, primary kidney disease, co-morbidities, nutritional status, Hb, ferritin, albumin, residual renal function and dialysis modality. Hazard Ratio (HR) for high ESA dose was 1.20 (95% CI 0.83-1.73) with a sequential Cox and 1.54 (95% CI 1.08-2.18) with a MSM. Patients treated with high ESA dose have a 1.2-1.5 increased risk of mortality. Our analyses support guidelines advising a conservative ESA dosing regimen, which carefully weighs the patients' benefits and risks. A manuscript with these analyses is in preparation.

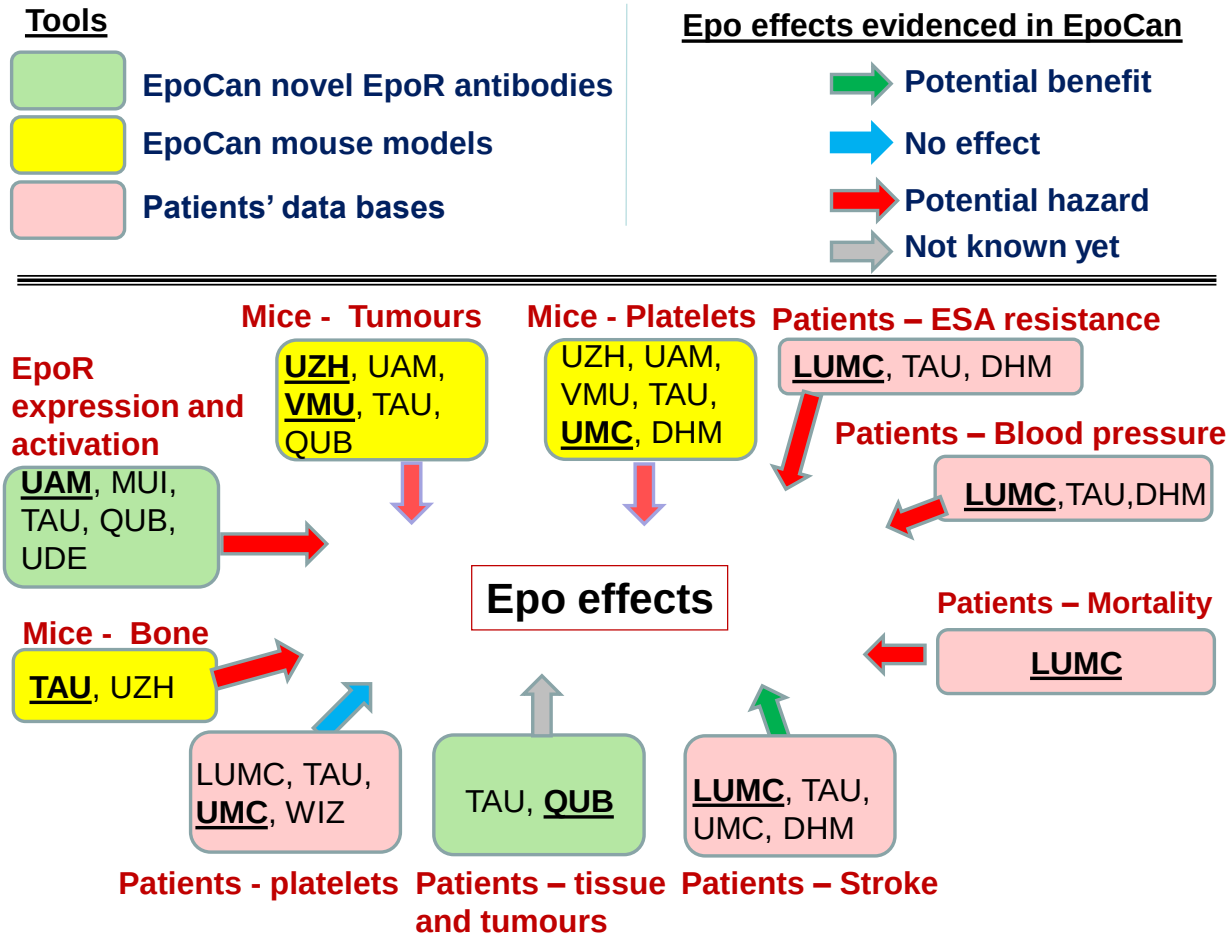
Effect of Epoetin on prothrombotic and proinflammatory changes

Plasma samples from patients from the REVIVAL 3 trial that received high dose, short term Epo were compared to placebo in acute myocardial infarction. No changes in proinflammatory cytokines were found in the Epo group, as compared to the placebo group. However, we found an increase in total factor FVII after treatment with Epo, that was not observed after placebo. However, the active form of FVII remained unchanged. Moreover, platelet activation (although under standard anti-platelet therapy) was not altered after treatment with EPO, as compared to placebo (Demetz et al. 2014). As a measure for thrombin generation *in vivo*, prothrombin fragment F1+2 were measured before and after treatment with Epo. No significant changes in circulating prothrombin fragment F1+2 between the groups were observed (Demetz et al. 2014). Analysis of long-term results of the REVIVAL-3 study showed no alterations in the clinical outcome in patients with AMI receiving high-dose, short term Epo as compared to the placebo group (Steppich et al. submitted).

To assess the effect of Epo on prothrombotic and proinflammatory changes in the circulating blood in patients with renal insufficiency plasma samples from dialysis patients were collected and analysed. Values of ADAMTS13 and Trap were significantly reduced in the patients receiving Epo. These findings suggest decreased platelet activation under therapy with Epo. Moreover, in patients receiving Epo after dialysis, a decrease in PF4, and thrombospondin was found, whereas significant increased MPO levels after dialysis were observed in the Epo group - as compared to patients not receiving Epo.

1.3.6: Results integration, analysis, conclusions and scientific management

This part of the project constantly monitored the progress of all arms, with the aim of optimizing experimental conditions, combining results from all research components (cell lines, mice models and patients' data bases) and translating EpoCan accumulated knowledge and data into clinical recommendations, based on the performed studies, optimizing benefit/risk ratio of Epoetin treatment



EpoCan recommendations and Open Questions

For cancer patients EpoCan consortium recommends to follow the Hb/Hct closely. Maintaining Hb<12/g/dl appears to be imperative and a minimal safety rule. In haematological neoplasms, such as MM and MDS, Epo administration appears to be safe. **In solid tumours** it is advised to adhere to the published guidelines of the National Comprehensive Cancer Network (NCCN), and the European Organization for Research and Treatment of Cancer (EORTC), and a combined initiative of the American Society of Clinical Oncologists (ASCO) and the American Society of Haematology (ASH).
In short, the ASCO-ASH guidelines from 2010 specify that rHuEpo is only to be given to avoid blood transfusions in patients with solid tumours who are chemotherapy-treated, or patients with lymphoproliferative disorders, or low risk MDS. They also emphasize the importance of following the Hb levels closely (maintaining between 10-12 g/dl), and stopping if the patients do not respond within 6-8 weeks. They recommend treating only those patients for whom a cure is not expected, and use the hormone only in patients in whom a curative approach is not a real goal – until additional research clarifies the picture.

Open questions, suggestions for future trials and experimental studies for Epo use in cancer
In order to address the question of whether rHuEpo treatment is beneficial or detrimental to the cancer patient, research must continue to identify EpoR with specificity, to determine whether and how it functions to enhance or inhibit tumour growth. Given the vast heterogeneity of cancer in general, even within specific tumour types, it is likely that Epo will be

found to be beneficial for some, and detrimental for others, as shown in the EpoCan mouse experiments (WP2). If that is so, then testing for the relevant genes or markers on the cell surface, or within the cell, could determine who would benefit from the medication. Finally, development of new ESAs that specifically enhance erythropoiesis, without affecting tumorogenesis, would help patients who suffer from cancer related anaemia - while not promoting tumour growth.
EpoCan findings on Epo associated bone loss strongly endorses close monitoring of bone parameters in Epoetin treated patients, and urge the need for deciphering the effects of Epo on bone parameters in Epo treated patients.

EpoCan recommendations *for Epo use in chronic kidney disease*
Taking everything together, **the one consistent finding of the anaemia-correction trials in CKD patients is a similar to worse overall mortality and event rate with higher Hb targets.** Since achieving survival benefit seems illusory and evidence for further improvement in quality of life is weak, higher Hb targets should not routinely be aimed for. However, since achieved higher Hb levels do not seem to be related with adverse events, it seems unlikely that the Hb levels are the cause of the unexpected higher event rate in the higher Hb arm of the anaemia-correction trials. Therefore, the higher ESA doses or ESA resistance remain a subject of debate. The current guidelines recommend treating with the lowest ESA dose possible to avoid blood transfusions. Given the first positive experiences with low dose ESA and especially the risk of inducing alloantibodies with recurrent blood transfusions in patients possibly waiting for a kidney transplant, this seems like a wise strategy; it is also in line with the recommendation for cancer associated anaemia (see above).

Open questions, suggestions for future trials and experimental studies for Epo use in chronic kidney disease
In contrast to oncology, ESA treatment is a common practice in CKD patients. This is possibly because the alternative of frequent red blood cell transfusions is even less appealing, especially with a risk for inducing alloantibodies in CKD patients waiting for a kidney transplant. An open question remains whether the adverse events can be attributed to the ESA treatment itself, only the higher ESA doses, or ESA resistance. As long as the mechanism is unrevealed, this debate will continue. Furthermore, special attention should be paid to geographic differences in anaemia treatment and outcomes [2, 4]} The two largest anaemia-correction trials were mainly performed in the USA, where patients are being treated with considerably larger ESA doses than in most European countries. It is therefore questionable if these results are generalizable to Europe. In the nearby future, the upcoming C.E. DOSE trial should answer if high ESA doses in CKD patients cause more events than low ESA doses. Hopefully, these results will provide us with further guidance regarding the anaemia management. Meanwhile, studies should focus on unravelling the underlying mechanism that is responsible for the higher event rate in the higher Hb target arms of the anaemia correction trials.

Recommendations for Epo use with respect to thrombosis and platelet function
Although no platelet activation or activation of coagulation by Epo was observed in the experiments conducted by EpoCan (WP4), the fact that Epo-overexpressing mice with an increased haematocrit develop thrombosis suggests particular caution in patients at higher risk of thromboembolic disease or with elevated haematocrit values. Further studies are required to determine the underlying mechanism. Thus, according to the KDIGO guidelines, we suggest ESA treatment only in patients with Hb<10g/dl. In contrast high dose, short term treatment is safe and does not alter platelet function, activation of coagulation or thromboembolic events. Yet, so far, the lack of effectiveness of this regimen in the clinical studies does not provide any treatment rationale.

1.4 Potential impact

(including the socio-economic impact and the wider societal implications of the project so far)

Clinical and scientific impact

The EpoCan project results lead to important new knowledge on major and serious adverse events caused by Epo treatment; i.e. the risk of increased mortality in Epo treated patients. EpoCan results will promote a better understanding of the role of EpoR in tumour biology and in CKD treated patients and will further yield improved pathological testing of patient samples that will lead to safer use of Epoetins.

The main outputs (non tangible) of EpoCan:

- Elaborated the understanding of EpoR signalling in cancer cells; Epo-R homodimerization is principally taking place in cancerous cells and that the formed Epo-R dimers are biologically active (i.e. respond to Epo stimulation). The data also show that sufficient JAK2 levels are a prerequisite for receptor activation.
- Elaborated the understanding of Epo contribution to cancer formation, propagation, and metastasis using novel animal models with spontaneous tumour formation:
    - Epo overexpression leads to a later onset of tumour formation suggesting that Epo might be anti-tumorigenic before a tumour cell generates. The deletion of EpoR in spontaneously developing tumours causes an earlier tumour onset confirming the result that EpoR might delay cancer initiation.
    - Epo overexpression results in faster tumour growth suggesting that Epo, as soon as a tumour has been formed, promotes tumour growth.
    - Epo injection did not lead to a change in survival of tumour-bearing animals, suggesting that very high levels of Epo might be needed to enhance tumour growth.
    - Animals with tumours lacking EpoR displayed induced tumour growth after Epo administration suggesting that Epo might promote tumour growth independently from EpoR expression in cancer cells
- Elaborated the understanding of Epo contribution to cancer formation, propagation, and metastasis, using tumour xenograft models carrying human breast and lung cancer cell lines:
    - Epo stimulates cancer cell growth in lung cancer cells (especially A549)
    - Epo affects metastatic potential of cancer cells
    - The loss of EpoR is associated with reduced tumour cell growth *in vitro* (MDA-MB-231 and A549) and also *in vivo* (A549) and suggests that EpoR plays an essential role – at least in the cell lines tested and this effect is independent of Epo injections
- Unravelled that **EpoR is broadly expressed in tumour cells including LLC**, originally planned as EpoR-deficient cell line relevant to assess the role of tumour stroma in Epo-driven protumoral activities.
- Unravelled that Epo has has no direct effects on platelet function.
- Demonstrated that in patients receiving high dose, short term Epo treatment in acute myocardial infarction there was no enhanced proinflammatory or prothrombotic changes in the circulating blood, and long term results showed the safety of this regimen of high dose Epo.
- Demonstrated that Epo stimulating agents (ESA) resistance is associated with increased mortality in both HD (haemodialysis) and PD (peripheral dialysis) patients.
- Demonstrated that in incident dialysis patients ESA was not associated with an excess of thrombotic events. On the contrary, in our data the association between ESA use and ischemic stroke seemed even protective. We found no clear association between ESA use and MI and no evident dose-response relation.
- Confirmed the hypertensive effect of ESA. However, it seems therefore questionable that the effect of ESA on blood pressure could contribute to an increased cardiovascular risk associated with ESA use in these pre-dialysis patients.
- Patients treated with high ESA dose showed a 1.2-1.5 increased risk of mortality. These results support guidelines advising a conservative ESA dosing regimen, which carefully weighs the patients' benefits and risks.

The main outcomes (tangible) of EpoCan:

- Generated and validated specific EpoR antibodies. Two rat monoclonal antibodies are valuable for comparative IHC studies in a wide range of cell lines and human tumour tissues.
- IHC studies have been performed using tissue microarray (TMA) samples from 519 cancer patients with lung, breast and ovarian oesophageal cancer.
- Novel mouse models with spontaneous tumour formation that over-express Epo. These models are valuable for the study of the involvement of Epo in cancer formation and propagation.

- EpoR silenced cells that will be valuable tools for research on the effect of Epo on cancer growth that is not mediated via the EpoR.
- Developed novel mouse models to investigate the effect of EPO on thrombosis and atherosclerosis.

Socioeconomic impact:

⇒ **Health care**

Even in developed countries, which have, relatively, stringent regulatory authorities, several drugs have been withdrawn from the market in the past few years, including: Alosetron (then returned to market with restrictions and a label warning), Troglitazone, propulsid, cerivastatin, rofecoxib, valdecoxib. Several other products, such as antidepressant and NSAIDs have received additional warnings and restrictions based on data captured from drug safety activities. In developing countries, where several million people are taking drugs for chronic diseases such as HIV/AIDS and tuberculosis, many of which are known to have serious **side effects and a narrow therapeutic window**, gathering safety data is imperative.
EpoCan results will provide knowledge and understanding that would result in revision of Epo treatment, thus reducing side effects and health care cost for cancer and CKD patients which are today the most economic burden in the western countries.

⇒ **Employment**

EpoCan has applied a cross-disciplinary research area, which promotes collaboration between biology (cellular and molecular), clinics, and computers. Thus, it represents promising technological fields for growth & sustainable employment in Europe. The novel models and antibodies developed in the project would become powerful tools alongside, or even over the existing methods, and hence EpoCan will contribute to increase job opportunities & competitiveness in biology (experimental and computational) and relevant industrial sectors of the European participants in EpoCan.

⇒ **Quality of life of the EU citizens**

Epo is used in treating anaemia resulting from chronic kidney disease and myelodysplasia, from the treatment of cancer (chemotherapy and radiation), and from other critical illnesses (heart failure).
After 25 years since the introduction of rHuEpo into clinical practice, and despite the broad clinical usage of Epoetins, there is not enough information on those drugs adverse reacting system.
Thus, EpoCan results and recommendations for Epo treatment enhance the knowledge on Epo adverse effects enabling a controlled treatment with a positive benefit to risk ratio. Numerous studies demonstrate that this treatment has a positive influence on the quality of life of the patients. Numerous studies showed that patients treated with chemotherapy and suffering from anaemia, treated with Epo demonstrated significant improvements in energy level, daily activities, and overall quality of life. The outcomes of the EpoCan project will result in a safe treatment with Epo increasing the quality of life of European citizens.

1.5 Main dissemination activities and exploitation of the results (about 6 pages)

Dissemination:

The Epocan consortium has allocated substantial efforts and resources for the purpose of dissemination of the project results within the scientific community and in the general public. The main efforts and dissemination activities are presented below.

Project Brochure:

The consortium has produced the project brochure. EpoCan Project Brochure was designed as a dissemination tool for the project. The brochure contains a general description of the project. The informative brochure promotes and describes the vision of the project, main objectives and outcomes as well as the project layout. The brochure also contains information about the project partners and their contact info. The brochure was used by the partners during their participation in conferences and meetings to disseminate the Epocan results.

Website construction and maintenance:

The EpoCan website (http://www.epocan.com/) was launched on March 2012 and has been updated regularly. The website is a library of all the project documents including all deliverables and presentations from the meetings. The website contains two main sections;

1. The public section that aims to introduce the EpoCan consortium, project concept, objectives and vision.
2. The partners' restricted section which offers an access to project records, e.g. submitted documents, deliverables, and meetings updated information including partners presentations and minutes. Every partner has received a private user and login password.

Presentations and posters at Conferences by EpoCan Consortium partners during M19-M36:

1. **TAU**: **Erythropoietin (EPO) exerts an anti-neoplastic activity: Characterizing EPO driven responses in the 5T33 multiple myeloma mouse model.** D. Neumann. *Leubeck 2012, Epo Meeting (oral)*
2. **TAU: Novel Erythropoietin-Receptor peptide antagonists.** Hiram-Bab S., Liron T., Souroujon M.C., Eisenstein M. and Neumann D. *Leubeck 2012, Epo Meeting (poster)*
3. **TAU: Benefits and Risks of Erythropoietin Treatment - Studies on the 5T33 Multiple Myeloma Mouse Model.** D. Neumann. *Essen, Germany 2012 HypoxiaNet Conference (Poster and oral)*
4. **LUMC: Erythropoiesis-stimulating agent resistance and mortality in Hemodialysis and Peritoneal Dialysis patients.** Suttorp MM, Hoekstra T, Rotmans JI2, Dekker FW. *WEON Conference*
5. **LUMC: ERA/EDTA conference.** 2012
6. **UAM: Acute VHL Gene inactivation induces cardiac HIF dependent erythropoietin gene expression.** *Leubeck 2012, Epo Meeting (Oral presentation).*
7. **UAM: THE ROLE OF THE HIF2α OXYGEN-SENSING PATHWAY ON mTORC1 ACTIVITY.** Ainara Elorza, Inés Soro-Arnaiz, Florinda Meléndez-Rodriguez, Glenn Marsboom, Alicia Vara-Vega and Julián Aragonés. *Essen, Germany 2012 HypoxiaNet Conference. (Oral presentation).*
8. **UDE: Analysis of the erythropoietin receptor dimer in different cancer cell lines by means of Fluorescence Resonance Energy Transfer.** André Bernardini, Ulf Brockmeier, Joachim Fandrey**.** *Leubeck 2012, Epo Meeting*
9. **TAU: Erythropoietin Receptor in endoplasmic reticulum stress – Maintenance of Surface Levels despite Reduction of Intracellular Receptor Levels** Dana Inbar, Orly Ravid, Constantinos Koumenis, Nathalie Ben-Califa, Fritz Grunert, John Thompson and Drorit Neumann. *Biennial Retreat of the Cancer Biology Research Center, ZEFAT 2013*
10. **TAU: Bone loss mediated by erythropoietin treatment in health and disease – from effect to mechanism** Sahar Hiram-Bab, Naamit Deshet-Unger, Mor Gross, Yankel Gabet, Moshe Mittelman Max Gassmann and Drorit Neumann *Gordon Research conference on Red Blood Cells 2013*
11. **UDE: Detection of Epo receptor homodimer activation in different cell lines by fluorescence resonance energy transfer microscopy.** *André Bernardini, Ulf Brockmeier, Joachim Fandrey. German Physiological society 2013 Heidelberg Acta Physiologica 2013; Volume 207, Supplement 694 :P017*

12. **WIZ: Big data vs Small data analysis.** Mira Marcus-Kalish. *IBS-EMR – the International Biometric society 2013.*
13. **TAU: Rational design of a novel Erythropoietin-receptor peptide antagonist.** Hiram-Bab S., Liron T., Souroujon M.C., Eisenstein M. and Neumann D. *CLINAM 2013 (meeting in Basel).*

14. **UDE: Investigation of erythropoietin receptor activation in living cells by Forster resonance energy transfer microscopy.** *A. Bernardini, U. Brockmeier, J. Fandrey. *German Physiological Society 2014.* Acta Physiologica March 2014 • Volume 210 • Supplement 695 P205
15. **QUB and EpoCan Consortium: Sensitive and Specific Antibody Probes Directed Against the Erythropoietin Receptor – From Basic Studies to Clinical Implementation.** Perry Maxwell, Florinda Melendez, Kyle B. Matchett, Julian Aragones, Nathalie Ben-Califa, Heidelinde Jaekel, Ludger Hengst, Andre Bernardini, Ulf Brockmeier, Joachim Fandrey, Fritz Grunert, Moshe Mittelman, Mohamed El-Tanani, Markus Thiersch, Edith Schneider-Gasser, Max Gassmann, David Dangoor, Terence Lappin, John Thompson, and Drorit Neumann. American Society of Hematology (ASH) meeting 2013.
16. **GVC and EpoCan Consortium: Sensitive and specific antibody probes directed against the erythropoietin receptor – from basic studies to clinical implementation.** HAH Meeting 2013. April 2014 Vienna.

17. **QUB: Erythropoietin (Epo) induces JAK/STAT-dependent proliferation of human cervical cancer *in vitro* and *in vivo*** Kyle B. Matchett , Tania V. Lopez , Perry Maxwell , Rebeca Lopez-Marure , Cecilia Aguilar , Zhanzhong Shi , John A. Thompson, Leticia Rocha-Zavaleta, Mohamed El-Tanani , Terry R. Lappin. *Irish Association for Cancer Research (IACR) meeting. 2013*
18. **LUMC: Erythropoiesis-stimulating agents and thrombotic events in dialysis patients** Marit M. Suttorp, Tiny Hoekstra, Gürbey Ocak, Anouk T.N. van Diepen, Ilka Ott, Moshe Mittelman, Ton J. Rabelink, Raymond T. Krediet, Friedo W. Dekker *ERA/EDTA meeting June 2014*
19. **UZH: The Impact of Erythropoietin on Cancer Growth.** Markus Thiersch, Maja Rütten, Johannes Vogel, Sophie Schober, Thomas Rülicke, Carla Rohrer-Bley and Max Gassmann. Tagung der FAchgruppe Physiologie und Biochemie, DVG Meeting Zurich, 2014; Award for best poster
20. **LUMC: Treatment with high dose of erythropoiesis-stimulating agents and mortality: a marginal structural model and sequential Cox approach.** Suttorp MM, Hoekstra T, Putter H, Dekker FW. *WEON Conference* 2014
21. **TAU: Erythropoietin reduces bone formation and stimulates bone resorption: new insights into endocrine regulation of bone remodelling.** Sahar Hiram-Bab, Tamar Liron, Naamit Deshet-Unger, Mor Gross, Salamon Avi, Moshe Mittelman, Max Gassmann, Martina Rauner, Ben Wielockx, Drorit Neumann and Yankel Gabet. To be submitted to conference of the American Society for Bone and Mineral Research.
22. **WIZ- "Technologies Enabling Personalized Medicine",** Mira Marcus-Kalish, CLINAM 7/ 2014, June 23-25 Bazel.
23. **TAU (UZH) Liver Macrophages (Kupffer cells) As Novel Targets of Erythropoietin**, Yasmin Ohana Haim, Naamit Deshet, Mor Gross, Nathalie Ben-Califa, Max Gassmann, Miriam C. Souroujon, Moshe Mittelman, and Drorit Neumann 56th ASH Annual Meeting and Exposition (December 6-9, 2014)
24. **LUMC: Treatment with high dose of Erythropoiesis-Stimulating Agents and Mortality***: A Sequential Cox Approach and Marginal Structural Model. M.M. Suttorp, T. Hoekstra, M. Mittelman, I. Ott, R.T. Krediet , F.W. Dekker, H. Putter. Poster American Society of Nephrology (ASN)*

Media broadcast:

1. **GVC: Aldevron to generate antibodies for erythropoietin research consortium** Press release in English and German announcing Aldevron´s participation in EpoCan. http://www.aldevron.com/resources/press/files/category-pr.php Dec 2011.

Scientific Papers published acknowledging EpoCan:

1. **TAU: Erythropoietin driven signaling and cell migration mediated by polyADP-ribosylation**. Inbar D., M. Cohen-Armon and D. Neumann. *Br J Cancer. 2012 Oct 9;107(8):1317-26.*
2. **UAM: HIF2α acts as an mTORC1 activator through the amino acid carrier SLC7A5.** Elorza A, Soro-Arnáiz I, Meléndez-Rodríguez F, Rodríguez-Vaello V, Marsboom G, de Cárcer G, Acosta-Iborra B, Albacete-Albacete L, Ordóñez A, Serrano-Oviedo L, Giménez-Bachs JM, Vara-Vega A, Salinas A, Sánchez-Prieto R, Martín del Río R, Sánchez-Madrid F, Malumbres M, Landázuri MO, Aragonés. *Mol Cell. 2012 Dec 14;48(5):681-91.*
   **Because of the relevance of this study, it has been highlighted in the Research Watch section of Cancer Discovery. "An Amino Acid Carrier Links the HIF2a Pathway and mTORC1 Regulation". Published OnlineFirst November 8, 2012; doi: 10.1158/2159-8290.CD-RW2012-195.*
3. **LUMC (TAU, DHM): Erythropoiesis-stimulating agent resistance and mortality in hemodialysis and peritoneal dialysis patients.** Marit M Suttorp, Tiny Hoekstra, Joris I Rotmans, Ilka Ott, Moshe Mittelman, Raymond T Krediet, Friedo W Dekker. *BMC Nephrol. 2013 Sep 25;14:200*
4. **LUMC (TAU, DHM): Effect of erythropoiesis-stimulating agents on blood pressure in pre-dialysis patients.** Suttorp MM, Hoekstra T, Mittelman M, Ott I, Franssen CF, Dekker FW. PLoS One*. 2013 Dec 31;8(12)*
5. **DHM (LUMC,UMC,TAU): The influence of Erythropoietin on platelet activation, thrombin generation and FVII/active FVII with AMI .** Gabriele Demetz MD, Magdalena Laux, Armin Scherhag, Tiny Hoekstra, Marit M Suttorp, Friedo Dekker, Marc Roest, Mira Marcus-Kalish, Moshe Mittelmann, Ilka Ott. *Thrombosis Journal 2014 AUG, 12:18 doi:10.1186/1477-9560-12-18*
6. **LUMC, (TAU,DHM): Erythropoiesis-stimulating agents and thrombotic events in dialysis patients.** Marit M Suttorp, Tiny Hoekstra, Gürbey Ocak, Anouk TN van Diepen, Ilka Ott, Moshe Mittelman, Ton J Rabelink, Raymond T Krediet, Friedo W Dekker. *Thrombosis Research. Available online 1 Aug 2014. DOI: 10.1016/j.thromres.2014.07.030*
7. **TAU, QUB, UAM, UDE, UZH, MUI, GVC: Novel Antibodies Directed against the Human Erythropoietin Receptor: Creating a Basis for Clinical Implementation.** *British Journal of Haematology.*

Manuscripts submitted:

1. **UMC: Recombinant EPO has no direct effect on platelet reactivity.** Submitted to British Journal of Haematology.
2. **TAU (UZH): Erythropoietin directly stimulates osteoclast precursors and induces bone loss** Sahar Hiram-Bab, Tamar Liron, Naamit Deshet-Unger, Mor Gross, Salamon Avi, Moshe Mittelman, Max Gassmann, Martina Rauner, Ben Wielockx, Drorit Neumann and Yankel Gabet ***(under revision for FASEB)***
3. **UDE**: **A flexible real-time system to acquire and analyze sensitized emission FRET microscopy images**. A. Bernardini, C. Wotzlaw, H.-G. Lipinski, J. Fandrey.

Manuscripts in preparation:

1. **UDE: Investigation of erythropoietin receptor activation in living cells by Forster resonance energy transfer microscopy.** *A. Bernardini, U. Brockmeier, J. Fandrey. et al
2. **LUMC: Treatment with high dose of Erythropoiesis-Stimulating Agents and Mortality: A Sequential Cox Approach and Marginal Structural Model.** M.M. Suttorp, T. Hoekstra, M. Mittelman, I. Ott, R.T. Krediet , F.W. Dekker, H. Putter. Poster American Society of Nephrology (ASN)

Exploitation:

Three major exploitable results have been identified by the Epocan consortium:

1. Novel specific EpoR antibodies generated within EpoCan. These antibodies are valuable for research purpose and would potentially be used as markers for patient stratification.
2. A gallery of novel mouse models with spontaneous tumour formation that over-express Epo. These models are valuable for the study of the involvement of Epo in cancer formation and propagation.
3. The profound understanding of the EpoR pathways and potential clinical side effects generated by the consortium.

The following exploitation strategy has been formulated for each of the results described above.

Novel specific EpoR antibodies*:*

Partner GVC is planning to sell two of the antibodies as reagents (GM1201 and GM1202). These will each be sold for €275/100 ug purified antibody. GM1202 is already listed on the Aldevron website and the data sheet for GM1201 is currently being finalized. As these antibodies were generated by different technologies, their application areas differ and thus can both be valuable tools. It is difficult to estimate the overall income/year, but it is predicted that each antibody will generate approx. €10,000/year. A third antibody may also become valuable as a reagent. This antibody currently does not have the GM number, as it is still being evaluated. There are indications that this antibody recognizes a variant of EpoR in some cancer cell lines.

Novel mouse models with spontaneous tumour formation that over-express Epo:

Several novel mouse models that are valuable for the study of EpoR involvement in cancer were generated. These models will serve the partners to generate valuable scientific findings and to generate scientific publications and strengthen their position in the Epo field.

Understanding the EpoR pathways and potential clinical side effects:

The knowledge generated within the Epocan paved the way of the consortium to generate a new proposal to respond to the Horizon 2020 call, PHC3- 2015 understanding common mechanism of diseases. Thus, Epocan tools generated within the project (antibodies and animal models) allowed the partners to envision the (low or high) EpoR signalling as a common mechanism of disease and apply for funding to validate this concept.

1.6 Project public website, and contact details.

Project website address: **http://www.epocan.com/**

List of beneficiaries:

Project Coordinator: Prof. Drorit Neumann, Tel Aviv University, Department of Cell and Developmental Biology. Tel: + **972-3-640-7256.** E-mail: **histo6@post.tau.ac.il**