FINAL PUBLISHABLE SUMMARY REPORT

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List of partners

No	Participant legal name	Short name	Country	Organization type
1	Pieris Pharmaceuticals GmbH[1] (former Pieris AG)	PIERIS (coordinator)	Germany	SME end-user
2	Technical University of Munich	TUM	Germany	University
3	Medical University of Innsbruck	MUI	Austria	University
4	Radboud University Nijmegen Medical Centre	RUNMC	The Netherlands	University
5	Covance Laboratories Ltd	COV	United Kingdom	Large
6	Antitope Ltd	ATOP	United Kingdom	SME
7	Fujifilm Diosynth Biotechnol-ogies Ltd.	FDB	United Kingdom	Large
8	Coriolis PharmaService GmbH[2]	COR	Germany	SME
9	FGK Clinical Research GmbH	FGK	Germany	SME
10	ConsulTech GmbH	CT	Germany	SME
11	Coriolis Pharma Research GmbH[3]	CPR	Germany	SME

[1] PIERIS changed its legal form and name
[2] COR had been a partner and left the consortium
[3] CPR is a new partner and a replacement for COR

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4.1 Final publishable summary report

4.1.1 Executive Summary

The EUROCALIN (EUROpean consortium for antiCALINs as next generation high-affinity protein therapeutics) Project was a 52 months EU FP7 project on-going from 2011 to 2015. It brought together 10 partners from research and development centres, small and medium-sized enterprises (SMEs) and industry from across the EU. The aim of the EUROCALIN project has been to develop, manufacture and clinically test an Anticalin (PRS-080) specific for hepcidin, a small peptide circulating in human blood that is considered to be a key regulator of iron homeostasis and, therefore, an important target for the treatment of multiple types of anaemia.

PRS-080 was tested in preclinical research experiments and the drug substance was produced at clinical grade. After the development of the drug formulation and the toxicology testing, the drug was finally tested in a first-in-man Phase I clinical trial. A flow chart showing the various stages of the EUROCALIN project is shown in Figure 1.1.

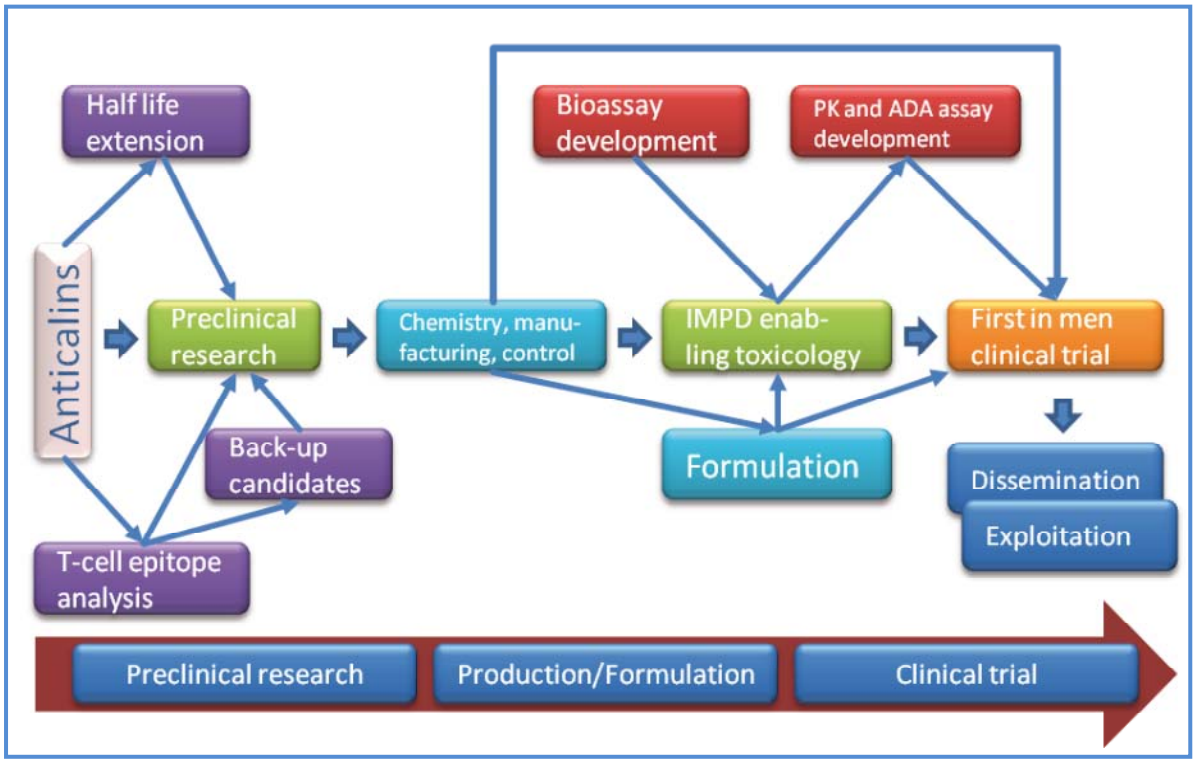

Figure 1.1: Flow chart of EUROCALIN project

Valuable experience for future clinical trials was gained through the development and manufacturing of the drug and of analytical bioassay systems for this novel high-affinity scaffold protein therapeutics. The Phase I clinical trial of the EUROCALIN project was completed successfully and the project was concluded only with a short prolongation of 4 months.

The research results from the EUROCALIN project have been disseminated nationally (in the countries of the project partners) and internationally through journal and conference papers and a podium presentation. Information from the project has been made available to academia and the pharmaceutical and healthcare industry.

4.1.2 Summary description of project context and main objectives

Anticalins are genetically engineered human lipocalins and represent a novel, non-immunoglobulin, next generation therapeutic protein class designed to bind and antagonize a wide spectrum of ligands. Anticalins exhibit high structural plasticity and are hypervariable in terms of conformation, length and sequence when compared to each other, reflective of the variety of observed binding specificities of natural lipocalins for vitamins, hormones or secondary metabolites. Showing picomolar target affinity and exquisite specificity, Anticalins also exhibit robust biophysical properties and can be produced at low cost in microbial expression systems, as they do not require post-translational modifications to the same extent as antibodies.

Only a relatively small number of sequence changes in the binding loops of a given human lipocalin, which are naturally non-immunogenic, are necessary to generate high affinity Anticalins, limiting a patient's risk of immune response to the therapeutic protein. Anticalins, therefore, represent a highly promising human, non-immunoglobulin scaffold, providing high-affinity therapeutic drugs with advantages for many medical applications.

The Anticalin drug candidate PRS-080 on which the project was focused binds to and antagonizes hepcidin, a small peptide circulating in the human body thought to be a key negative regulator of iron homeostasis. Iron is important for the generation of haemoglobin and red blood cells (erythrocytes) which transport oxygen to all body tissue. Hepcidin regulates the up-take of dietary iron and allows for optimal recirculation of iron from dying red blood cells and utilization of stored iron and, thus, affects generation of new red blood cells. Elevated hepcidin enhances iron storage and reduces iron availability, resulting in reduced haemoglobin and red blood cell production. Antagonizing such inappropriately elevated levels of hepcidin promises to play a key role in normalizing haemoglobin plasma levels, by increasing the systemic availability of iron in the circulation. PRS-080 will be tested for the treatment of anaemia in association with chronic and inflammatory diseases (anaemia of chronic disease, ACD).

Anaemia is a condition marked by a deficiency of red blood cells or of haemoglobin in the blood, resulting in pallor and weariness. The major pathophysiological factor in ACD is retention of iron, rendering the metal ion unavailable for generation of red blood cells (erythropoiesis). Anaemia in patients with chronic kidney disease (CKD) – a serious indication affecting approximately 4.5 million European patients - is often treated by administering erythropoiesis-stimulating agents (ESA) such as Erythropoietin (EPO).

Many patients with CKD and anaemia can be effectively treated with Erythropoiesis Stimulating Agents (ESA). However, around 10 % of patients (~ 150,000 patients in the EU) are hypo- or non-responsive to ESA, leaving them without an effective treatment option. An unwanted role of ESA resistance has been demonstrated by the results of clinical trials that reported an increased mortality or morbidity in patients who received high doses of ESA but did not reach the targeted haemoglobin plasma concentration. Increased mortality rates in

anaemic cancer patients treated with high ESA doses have recently been observed, raising yet additional safety concerns. In addition, the urgent need for new therapies to combat ACD is reflected by the fact that a large fraction (about 40-50 %) of anaemic cancer patients are hypo or unresponsive to ESA therapy (~740,000 patients in the EU). Therefore, the development of alternative treatment strategies for ACD, specifically in patients with chronic kidney disease and cancer, is of utmost importance.

The exact mechanism of ACD is not fully understood but current results strongly suggest that hepcidin plays a very important role in regulating the iron balance in the body (see Figure 2.1). Hepcidin is synthesized in the liver, enters the blood stream and binds tightly to the iron channel protein ferroportin, which is found amongst others on the surface of reticuloendothelial cells. These cells store iron. If hepcidin therefore binds to ferroportin it prevents the secretion of iron, thereby functionally reducing iron absorption. As a consequence, the body cannot effectively use iron to make new red blood cells and the number of healthy new red blood cells gradually falls.

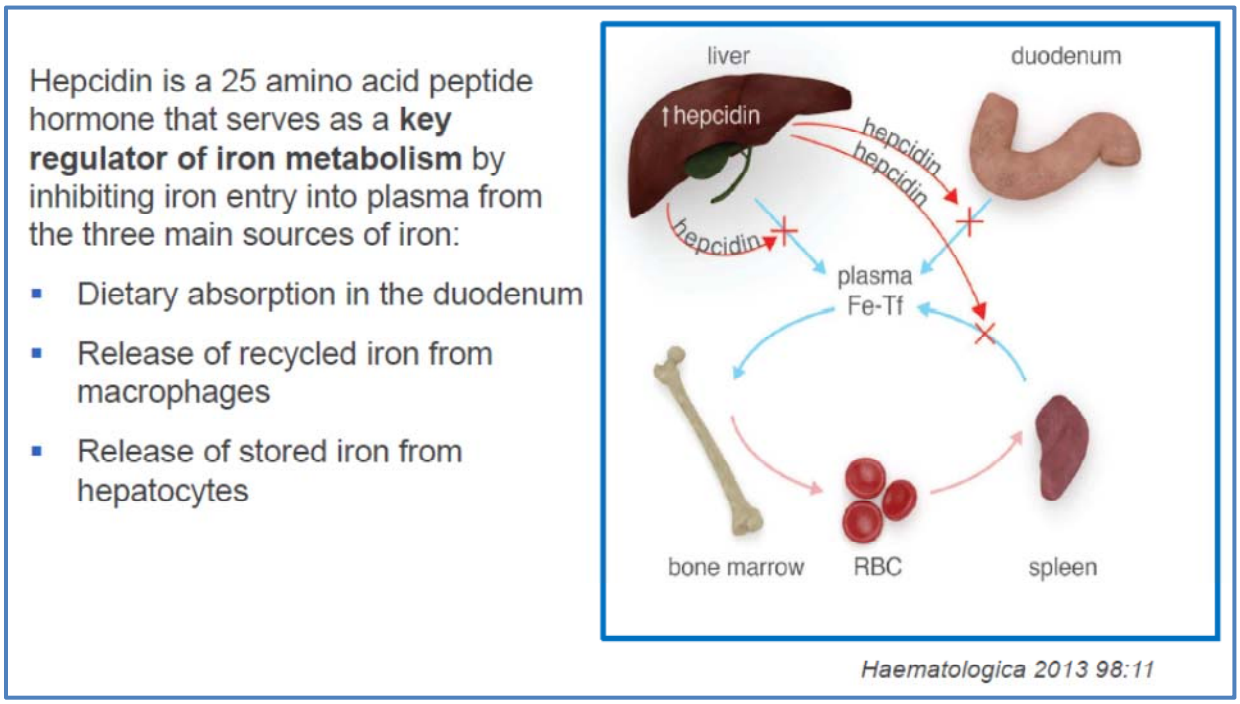

Figure 2.1: Hepcidin Plays a Central Role in Iron Metabolism

The consortiums aim is to develop a new treatment for ACD in which hepcidin is blocked by the PRS-080 Anticalin (see Figure 2.2). Thus, inhibition of hepcidin is intended to reactivate ferroportin and utilize internal iron sources and to increase erythropoiesis, with normalization of hemoglobin levels and reversal of anemia. The objective of the consortium is to develop this drug candidate from candidate selection to the completion of clinical Phase I study. The consortium's activities and objectives include (i) the selection of the drug candidate based on the human lipocalin NGAL (neutrophil gelatinase-associated lipocalin) scaf-

fold, (ii) a method for optimizing its pharmacokinetic profile, e.g. its plasma half-life, (iii) the analysis of the immunogenicity profile of the candidate with the objective to reduce or eliminate this, (iv) the evaluation of the therapeutic approach in animal models of anemia, (v) the establishment of suitable assays for the analysis of hepcidin (total and free) in animals and in humans, (vi) the evaluation of the safety of the drug candidate in good laboratory practice (GLP) toxicology studies, (vii) the development of a manufacturing process and an appropriate formulation for storage and clinical use of the drug candidate, (viii) the manufacture and stability analysis of drug candidate material for the conduct of a phase I clinical study, and (ix) the conduct of a phase I clinical study investigating the safety, pharmacokinetics, immunogenicity, hepcidin neutralization (target engagement) and biological activity of the drug candidate in healthy human subjects.

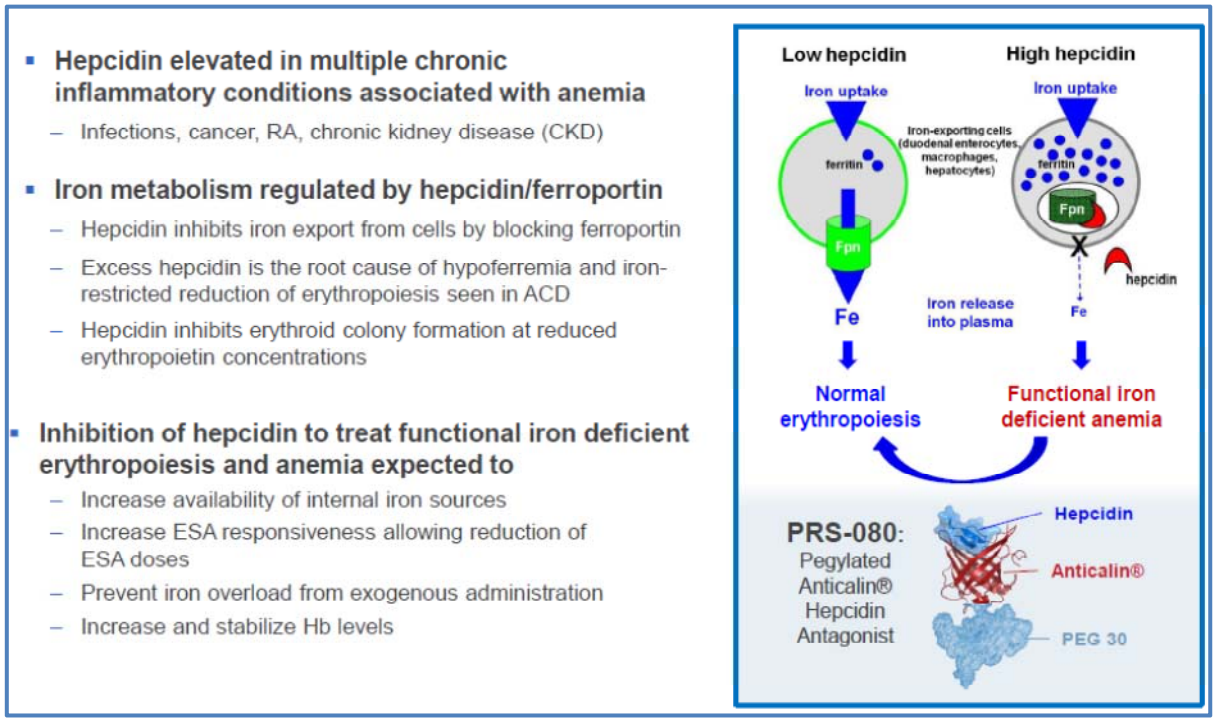

Figure 2.2: Antagonizing Elevated Hepcidin Levels in Anaemias of Chronic Disease

4.1.3 Main Scientific & Technical results/foregrounds

To achieve the overall goal of completing successfully a clinical Phase I study, the consortium members addressed and answered several key scientific and technical questions, which were broken down into the following specific and interdependent work packages.

WP1 Preclinical research

In this work package (WP), cell culture studies and animal models were used to study the short and long term metabolic effects following the interaction of PRS-080 with hepcidin towards systemic and organ specific iron homeostasis. Furthermore, we examined pharmacokinetics (PK) and pharmacodynamics (PD) of Anticalins to define optimal dosage and dosing intervals.

To investigate the activity of PRS-080 in cell culture, a cellular model was employed that allowed monitoring of the expression of ferroportin on the cell surface. Hepcidin down-modulated or reduced the expression of ferroportin in its known manner. PRS-080 added to the cell system was then able to bind to and block hepcidin, thus, preventing the down-modulation of ferroportin. This assay system was shown to demonstrate the potency of PRS-080 *in vitro*.

The PRS-080 Anticalin bound human hepcidin with high affinity but was only poorly reactive against hepcidin in rats. In order to perform preclinical studies in rats, a so called surrogate Anticalin was employed that had similar activity against rat hepcidin as the PRS-080 molecule against human hepcidin. First activity *in vivo* of Anticalin based hepcidin binding was demonstrated in normal rats with this surrogate Anticalin, fused to an albumin binding domain (albumin binding is used to obtain half-life extension of Anticalins, see WP2). 24 hours after administration of the surrogate Anticalin, increased serum iron levels and decreased iron retention in the spleen was observed. The activity of the surrogate Anticalin could be correlated to hepcidin binding. These results also guided on the dose and treatment schedules that were investigated next in a rat disease model of anaemia of chronic disease (ACD).

ACD can be provoked in rats by administration of bacteria derived inflammatory components. The inflammatory response elevates the production and release of hepcidin by the liver, which then increases iron storage in macrophages, followed by reduced erythropoiesis and anaemia. Administration of the surrogate rat-hepcidin specific Anticalin, fused to an albumin binding domain, as in normal rats increased serum iron and reduced iron retention in the spleen but also increased haemoglobin to normal levels in these diseased animals. The analysis of erythroid progenitor cells in the bone marrow further supported the finding of increased erythroid cell maturation upon hepcidin inhibition and increased iron availability. These studies demonstrated a treatment effect by antagonizing hepcidin with the surrogate Anticalin in a relevant disease model. Studies investigating different dosing schedules and

combination treatment with erythropoietin were carried out and further supported and guided the clinical development path of PRS-080.

In addition, the selected development candidate, PRS-080-PEG30 was investigated in WP1 in order to establish the dose-response relationship in animals and to guide the doses to be investigated in the planned clinical study (WP10). Different doses of PRS-080-PEG30 were administered and the resulting serum iron response monitored. The minimal biological effect level (MBEL) was determined. Taken together, these results provided a rational for recommending a starting dose and specific dose escalation steps for the first-in-man study.

WP2 Half-life extension

Anticalins as small proteins are cleared rapidly via renal filtration. However, their plasma half-life can be prolonged e.g. by genetic fusion with an albumin binding domain, fusion to human serum albumin (HSA) or by site directed conjugation with polyethylene glycol (PEG). Conjugation with PEG has the advantage that different types of PEG are known (length, degree of branching) so that the pharmacokinetic behaviour and half-life can be varied over a range as best needed for the intended therapeutic approach. This provided the opportunity to test and select for the most suitable conjugation and half-life extension in preclinical models.

First, we demonstrated that neither PEGylation with different size PEG polymers nor recombinant fusion with HSA impacted the binding affinity nor cell based potency of the Anticalin PRS-080. PRS-080 conjugates with PEG 12, PEG20, PEG30 and PEG40 were generated and tested. Single dose studies were performed in three animal species. The derived findings were then extrapolated to humans using the so called allometric scaling method. Exploratory safety studies were then performed excluding any adverse events produced by these conjugates.

Based on the pharmacokinetic data, a model was developed that allowed to study the relationship of drug concentrations and half-life with pharmacologic effects (hepcidin reduction). The model predicted that a prolonged half-life over the natural Anticalin half-life was desired but that further increasing the half-life would not contribute more to prolonging hepcidin reduction. In contrast, a molecule with a half-life similar as or longer than that of the PEG40 conjugate would result in relatively high and unwanted concentrations of circulating PRS-080-hepcidin complexes.

The pharmacologic activity of the differently PEGylated PRS-080 molecules was then tested in a relevant animal species. A single administration of either conjugate transiently elevated serum iron levels. The concentration over time profiles increased with PEG 12, PEG20 and PEG30 conjugates but were not further increased with the PEG40 conjugate. The PRS-080-PEG30 conjugate showed the best results confirming also the simulations performed with the above model. Based on these data, the PEG30 conjugate was selected as the develop-

ment candidate for further studies (WP4, WP5, WP8). The HSA-fusion molecule, although also active *in vivo*, was not selected, due to a half-life in the range of the PEG40 conjugate and its non-superior activity *in vivo* over PRS-080-PEG30.

WP3 T-cell epitope analysis

To assess whether or not PRS-080 has an immunogenic potential and possibly generates an immune response, the protein was analysed in T activation assays. PEGylated PRS-080 and the matching wild-type lipocalin (NGAL) protein were studied together with monoclonal antibodies as reference molecules. These reference molecules were characterized before in the T cell activation assay and were selected based on their known clinical risk of immunogenicity (low risk and high risk antibody selected for this study). PRS-080 showed the same low level reactivity as observed with the low-risk reference antibody. Furthermore, PRS-080 and the NGAL behaved similarly, demonstrating the lack of additional immunogenicity over the natural protein. Based on these positive results, further engineering of PRS-080 with the aim to eliminate immunogenic epitopes was not necessary. Thus, the current PRS-080 molecule was selected as development candidate.

WP4 Chemistry, manufacturing and control

The aim of this work package was to establish a robust upstream and downstream process for the current good manufacturing practice (cGMP) production of the selected human hepcidin-specific lead Anticalin capable for supplying phase I clinical trials. The process included fermentation, downstream, the modification of the Anticalin for half-life extension, formulation of bulk drug substance and fill & finish. We were able to develop a GMP production process for PRS-080 in a scalable and cost efficient bioprocess.

An *E. coli* expression study was performed from which the PRS-080 encoding plasmid and the *E. coli* strain was selected. Next, a bacterial master cell bank was generated and subsequently characterized. An upstream and downstream process was developed. The fermentation conditions were optimized at small scale and different methods for releasing PRS-080 from the producer cell and the clarification of the production broth were tested. Analytical methods were set-up, qualified and subsequently validated in order to study and confirm the quality of PRS-080 through-out the manufacturing process (in-process controls and quality determining assays for product characterization). They were designed to characterize PRS-080 but also to test for known impurities such as host cell deoxyribonucleic acid (DNA) and protein as well as endotoxin. Further down-stream process steps were based on column chromatography. Different binding and release conditions were tested to come to an optimal purification scheme. The conditions for PRS-080 PEGylation were optimized by including analytical characterization of precursor, the intermediates and the PEGylated molecule. The

PEGylated and purified PRS-080 was dissolved in the final formulation developed under WP5 and subjected to a thorough analytical characterization.

The process was then scaled up to 100L for process demonstration and generated drug substance for a non-GLP dose range finding toxicology study. Further scale-up to 300L was performed and an engineering batch manufactured. The batch demonstrated the process capabilities and product characteristics as specified. Material from this batch was used in the 4 week GLP toxicology study (see WP8). A second batch was produced under identical conditions and at GMP. PRS-080 from this batch passed the release testing and was then filled under aseptic conditions into the final container. Samples from the engineering batch and the GMP batch were subjected to long term stability studies (see WP5).

A suitable placebo material was produced at GMP as well and filled into the same containers as PRS-080. Both, GMP produced placebo and PRS-080 were used in the clinical study (see WP10).

WP5 Formulation

The main task of this work package was to develop a stable parenteral formulation for intravenous administration of PRS-080 for pre-clinical and clinical studies.

We set up stress conditions to be used during analytical and formulation development; furthermore, we developed, selected and validated methods that indicate stability and used pre-formulation screening to identify possible stable formulation conditions (e.g. protein concentration, pH, buffer, ionic strength, excipients) as the basis for the further formulation development. Using the different conditions and formulations, we were able to select specific conditions which were then tested at selected temperatures over time periods up to one year. Based on these studies, we were able to select a formation which was then used for further development.

PRS-080 manufactured at larger scale in the final manufacturing process (see WP4) was then used to conduct formal stability studies according to internationally accepted principles. Drug substance and drug product was stored in designated containers at the intended storage temperature. In addition, further vials were stored at elevated temperatures in order to accelerate potential processes leading to instability and to allow extrapolation to the intended storage conditions. Analytical methods indicating stability of PRS-080 were established and validated for use during the stability study. Stability criteria were established and for each parameter specific limits for acceptance were set.

At pre-defined time points, samples were pulled and analysed according to the pre-established protocols. Until now, the results of all analyses were within the pre-set acceptance criteria, thus demonstrating the stability of PRS-080. According to these results, expiry dates were established for the PRS-080 batch intended for use in the clinical study. The clinical study was successfully completed within this time frame. Due to the excellent stability of

PRS-080, the study is continued and additional time points will be evaluated. According to the data, longer than current established storage time periods will be assigned to PRS-080 allowing its prolonged use.

WP6 & WP7	Pharmacokinetics and anti-drug antibody assay development; Biomarker assays

Development of a drug candidate requires the assessment of its concentrations over time in plasma of treated animals or humans. Therefore, we set-out to develop and validate GLP-conform analytical methods:

PRS-080 is a protein and ligand binding assays employing antibodies for trapping and detection of PRS-080 (called ELISA - Enzyme Linked Immunosorbent Assay) represent the suitable format for the detection and quantification of PRS-080. Such ELISA based assays for the detection of the specific Anticalin in plasma or serum of the various animal species and humans were developed to ensure that compound kinetics and exposure can be demonstrated. Furthermore, an anti-drug antibody (ADA) ELISA was developed to detect the presence of ADAs directed against the Anticalin. The ADA assay was used as an indicator of immunogenicity in humans during the first-in-man study.

The assays were then used to determine free and hepcidin-bound Anticalin as well as anti-PRS-080 antibodies in the nonclinical and in the clinical study (WP8 and WP10). All scheduled samples were analysed and the resulting pharmacokinetic parameters such as maximal concentration, area under the concentration time curve, the half-life, elimination rates, volume of distribution, etc. were calculated. There was a dose-proportionality of the pharmacokinetic parameters observed in the selected animal species. The half-life of PRS-080 was approximately 2 days. Results obtained in humans are summarized under WP10.

PRS-080 is intended to bind and neutralize hepcidin, thereby reducing the concentrations of circulating hepcidin. Thus, methods were established and employed to determine the concentration of hepcidin in plasma of animals and humans. When PRS-080 binds to hepcidin, it is functionally not active anymore, however, it still circulates in the blood stream in its complexed form with PRS-080. In order to distinguish the unbound or free hepcidin from the total hepcidin (the free and the PRS-080 bound) in a sample, we established an assay specific for free hepcidin. Together with the assay for total hepcidin, we were then able to measure free and total hepcidin. Both methods are based on the so called mass-spectrometry method where hepcidin is identified by its specific mass and quantified by comparing the signal with that of an internal standard. The assays were validated in order to assure reliability of the generated data.

Hepcidin specific assays were employed for the analysis of animal and human samples. In animals we were able to show that the concentration of total hepcidin increased following PRS-080 administration as expected. The complex stayed longer in the circulation, likely due

to its size and a resulting slower elimination, contributed so to higher concentrations. Free hepcidin, in contrast, was reduced or stayed at baseline levels following administration of PRS-080. The determination of free hepcidin in the clinical study was pivotal for demonstrating hepcidin neutralization upon PRS-080 administration and to obtain an insight into its regulation as a consequence of PRS-080 administration and the associated changes in serum iron levels.

WP8 IMPD enabling toxicology

Investigator Medicinal Product Dossier (IMPD): According to general guidelines for conducting clinical trials a formal GLP safety assessment program was required in order to advance into clinical studies in humans. This work package addressed the required safety studies and assisted in defining the safe starting dose and safety margins for the first-in-human clinical studies. Within this work package we also identified specific safety parameters that were needed to be monitored in the clinical studies and defined the inclusion and exclusion criteria for the first-in-human studies.

A 12 day non-GLP dose range finding provided evidence for safety of PRS-080 Anticalin drug substance and provided the basis for subsequent studies regarding dose and treatment schedule. Furthermore, PRS-080 plasma concentrations were established as well as the effects on circulating hepcidin levels and pharmacologic effects such as elevation of serum iron. A study addressing the distribution of PRS-080 was performed using radio-labelled drug substance. We did not observe any undue tissue accumulation of the radio-labelled drug and observed that PRS-080 was in part excreted by the kidney. Subsequently, a 28-day GLP toxicity study was performed at three PRS-080 doses administered every other day. No adverse events were observed up to the highest dose tested. A kinetic assessment was performed and the generation of anti-PRS-080 antibodies (ADAs) was investigated. These studies and the resulting good safety profile established the foundation for the first in man clinical study with PRS-080 which was subsequently completed successfully (see WP10).

WP9 Back-up and surrogate candidates

This work package was part of our risk mitigation strategy, but its results might also be used as a next generation development for an optimized PRS-080. For contingency purposes, we intended to develop a human and/or rat hepcidin-specific backup Anticalin with a similar or better pharmacological profile as compared to the actual PRS-080 lead candidate.

In addition, it was crucial to confirm that rat hepcidin-specific Anticalins, which are being used as surrogate molecules to validate the pharmacological approach, engage this peptide target in a comparable manner to the Anticalin specific towards human hepcidin that was chosen for clinical development.

We were able to solve the co-crystal structure of the corresponding Anticalin bound to rat hepcidin by means of X-ray crystallography. These structural data generated with the surrogate Anticalin should in principle be transferable to the therapeutic development candidate. It turned out that further affinity maturation of the human hepcidin-specific Anticalin as well as the surrogate Anticalin was not necessary based on the positive results from preclinical research, half-life extension, T-cell epitope assay and CMC (chemistry, manufacturing and control) development.

Informed by these structural data, also specific mutations were introduced into the clinical Anticalin candidate in order to abrogate hepcidin binding. This led to insight into the detailed interaction between PRS-080 and hepcidin and provided a more suitable negative control for preclinical studies with the human candidate as well.

WP10 First in men clinical trial

The planning, regulatory approvals, conduct, and evaluation of a single dose phase I study in healthy volunteers was the aim of this work package. The study was supposed to establish safety, lack of immunogenicity, tolerability, pharmacokinetic parameters, and dose-dependent target engagement. The results should define safe and potentially effective dose levels for a subsequent study in patients with anaemia of Chronic Kidney Disease.

We devised a clinical protocol for the planned Phase I study and generated the Investigator Brochure (IB) and the Investigational Medicinal Product Dossier (IMDP). The clinical trial application (CTA) containing these documents among others was filed with the German Bundesinstitut für Arzneimittel and Medizinprodukte (BfArM) and the concerned ethic committee (EC). Approval to conduct the study was obtained by both, the Agency and the Ethics Committee. PRS-080 was well tolerated and its biological activity, the release of stored iron and the increase of circulating serum iron, was established.

48 healthy male subjects were treated in this placebo controlled, double-blind Phase I study with ascending doses of PRS-080 in 6 cohorts at 0.08, 0.4, 1.2, 4.0, 8.0, and 16.0 mg/kg. Placebo or active treatments were administered by intravenous infusion over 2 hours. PRS-080 was well tolerated. 39 adverse events (AE) were reported during or after treatment in 22 subjects. All such AEs were mild or moderate and no Serious AE was observed. Headache was the most frequently observed AE (10 subjects). Otherwise, no association of AEs to specific organs and no apparent dose dependency or difference between placebo and active treatment were observed. Notably, no hypersensitivity or infusion reactions were noted and vital signs, body temperature and ECG were unchanged.

Pharmacokinetics of PRS-080 was consistent between dose cohorts and within subjects of each cohort. Maximal concentration (C_{max}) and area under the time curve (AUC) increased proportionally with dose. The terminal plasma half-life ($T_{1/2}$) of PRS ranged from 64 to 81

hours among dose cohorts (geometric mean). The volume of distribution was small with 49 to 65 ml/kg, consistent with a distribution mainly to the blood volume.

Administration of PRS-080 resulted in a decrease of free hepcidin which was observed already 1 h after start of infusion. PRS-080 administration induced a transient increase in serum iron concentration and transferrin saturation (TSAT), with both responses exhibiting a comparable time course and at doses of 0.4 mg/kg and higher. TSAT increased to > 90% in individual subjects. Serum iron concentrations reached about 50 µmol/l in individual subjects and did not further increase with dose. Importantly, the time period at which elevated serum iron concentrations and TSAT were observed increased with dose from about 18 h at 0.4 mg/kg to about 120 h at 16 mg/kg PRS-080. This is reflected by an increase of the AUC of the serum iron response relative to baseline and placebo.

The clinical strategy was further discussed with external experts. It was concluded that subsequent to the single ascending dose Phase I study in healthy subjects, a study in chronic kidney disease patients (single dose, followed by repeated dose) represents the best path to generate first meaningful data in a relevant patient population. Based on the excellent safety profile of PRS-080 and its pharmacological activity, the project will now be continued accordingly.

The scientific technical goal of EUROCALIN was achieved successfully. A candidate lead Anticalin was developed and characterized that demonstrated efficacy in animal models of ACD and successfully passed clinical phase I testing with the potential as a safe, effective, and novel medicine for this disease.

4.1.4 Potential impact

The major pathophysiological factor in Anemia of Chronic Disease (ACD) is retention of iron in macrophages, rendering the metal ion unavailable for erythropoiesis. Hepcidin is upregulated in ACD patients and was identified as the key mediator in the development and manifestation of ACD by blocking iron liberation from macrophages and enterocytes. An Anticalin (PRS-080) that specifically binds to and blocks hepcidin and, thus, normalizes iron utilization for erythropoiesis was developed in this project and successfully tested in a first clinical study. These drug development activities had or have implications on (i) the Anticalin technology itself, (ii) the understanding of hepcidin physiology and effects of hepcidin inhibition, and (iii) the future treatment of patients with ACD.

The technical development of PRS-080 within this project contributed to the better understanding of Anticalins and their potential use as a new drug class and resulted in the initiation and progression of new drug development projects. Different technologies that allow to modify the pharmacokinetic properties and the plasma half-life of Anticalins were studied, including conjugation to polyethylene-glycol (PEG) and fusion to albumin or to an albumin-binding moiety. Conjugation to PEG-of 30 kD size (PEG30) was found to be optimal for the intended inhibition of hepcidin in patients and was thus selected. The knowledge gained here impacts other Anticalin-based drug development projects and allows to fine-tune the pharmacokinetic properties depending on the pharmacological mechanism. The good safety properties, the knowledge on biodistribution and immunogenicity gained as well as the excellent tolerability observed in humans further supports and broadens the potential use of Anticalin based drugs for other indications. The know-how obtained during the development of a manufacturing process demonstrated the advantages of an *E. coli* based process and its suitability for manufacture of high quality drug products. In addition, through scientific advice meetings at the German Regulatory Agency (BfArM) and through the review of clinical trial application documents by the Agency and by Ethic Committees, PRS-080 and the Anticalin technology obtained regulatory exposure and input further qualifying Anticalins as a drug class. These advancements of PRS-080 and the Anticalin technology formed the basis for alliances with international pharma companies and the initiation and progression of additional drug development programs with them that utilize Anticalins for the treatment of diseases with unmet medical need.

The scientific results obtained during the project strongly progressed the knowledge about the use of hepcidin as drug target and antagonizing its activity as a modality to increase iron utilization and promote erythropoiesis. This know-how will help to advance the clinical development of PRS-080 and will also contribute to other drug development approaches in this field. The nonclinical models employed outlined the clinical potential of the Anticalin based anti-hepcidin treatment. Furthermore, the results obtained helped to guide the dosing and treatment schedule with regard to beneficial effects from iron mobilization on erythropoiesis while avoiding possible side effects from iron overload. New methodologies developed

during the project now allow better analysis and understanding of erythroid cell differentiation in the bone marrow, its inhibition by iron deficiency leading to anaemia and its normalization by hepcidin inhibition. New methods were developed to investigate hepcidin in the presence of the inhibitory Anticalin. To our knowledge, this made it possible for the first time to investigate remaining active (or non-neutralized) hepcidin after antagonist administration. It became possible to follow hepcidin levels and correlate them to the effects on iron metabolism. In particular the employment of these methods during the clinical study with PRS-080 provided a new insight into the interplay of iron and hepcidin regulation and the effects of neutralizing hepcidin. These results will benefit the further development of PRS-080 and its utilization in treating anaemia in ACD patients. Moreover, medical treatments aiming at iron and hepcidin metabolism and interfering with iron deficiency or overload will benefit in general.

Further development of PRS-080 will focus on the treatment of anaemia in patients with advanced stages of Chronic Kidney Disease (CKD), including CKD stage 5 patients. These patients have adequate stores of iron but this iron is not efficiently incorporated into red blood cell precursors through recombinant erythropoiesis stimulating agents (rESAs) and iron supplements. This imbalance in iron metabolism is a result of a high level of circulating hepcidin in the blood stream (see also Figure 2.2).

Many patients with CKD and anaemia can be effectively treated with ESA‘s. However, around 10 % of patients (~ 150,000 patients in the EU) are hypo- or non-responsive to ESA, leaving them without an effective treatment option. In the US there are about 640,000 CKD stage 5 patients, among which about 70% are anaemic. About 20% of the anaemic CKD5 patients or 90,000 patients in the US are believed to qualify for anaemia treatment with PRS-080. Existing therapies are limited in that they do not have an impact on hepcidin or, in the case of ESAs, patients often become resistant to the therapy. Furthermore, an unwanted role of ESA resistance has been demonstrated by the results of clinical trials that reported an increased mortality or morbidity in patients who received high doses of ESA but did not reach the targeted haemoglobin plasma concentration. Increased mortality rates in anaemic cancer patients treated with high ESA doses have recently been observed, further stressing the safety concerns connected to treatment with high ESA doses.

The Anticalin PRS-080 was engineered so that it binds to hepcidin and reduces the impact of hepcidin’s negative regulation on iron mobilization which is believed to be the underlying cause of iron deficient anemia in CKD patients. It is believed that by blocking the actions of hepcidin, PRS-080 will serve to address anemia by mobilizing iron for incorporation into red blood cells. In patients suffering from anemia of CKD, hepcidin is frequently produced by the body in abnormally large amounts. Therefore, we believe that the best way to inhibit its function is to administer an inhibitor frequently, such as once a week. Our approach will use PRS-080 in connection with a conjugated PEG30 molecule, potentially allowing the drug sufficient residence time. Once coupled to PEG30, PRS-080 is intended to have a half-life that will be optimally suited for dosing anemic patients with CKD. In contrast, antibodies typically

have a half-life of two to three weeks. Such a long half-life renders antibodies unsuitable for frequent administration and elimination of a circulating target protein like hepcidin because such antibodies tend to accumulate the target after binding due to their own long residence time in the body, with the associated risk of bound hepcidin being released by antibodies that are still circulating in the blood. Thus, the optimized half-life of PRS-080 is seen as a competitive advantage over other hepcidin binders, e.g. antibodies.

PRS-080 has the potential to become a new and effective treatment of anemia in patients with CKD and other forms of ACD who do not respond well to current treatments. The new treatment mechanism involving hepcidin blockade is believed to address the root cause of iron deficient anemia in the respective patients. This has the potential to prevent iron overload from exogenous administration and to increase ESA responsiveness allowing reduction of ESA doses. Both of these effects are expected to increase and stabilize hemoglobin, thereby preventing anemia and the associated morbidities. Given the high number of patients with high medical need, this new therapy has the potential to benefit a large patient population.

4.1.5 Main dissemination activities and exploitation of results

Throughout the lifetime of the project the partners were active in sharing the progress and the acquired knowledge with the scientific community and the interested public. Beneficiaries published 2 peer reviewed scientific papers and presented (either orally or by poster) at 49 conferences, trade shows or through articles in newspapers. Furthermore 10 press releases were used to inform the public on the start, the progress and the results of the EUROCALIN project. Press releases on important findings further supported business development activities.

All project related information was made publically available via the project´s website www.eurocalin-fp7.eu (new: eurocalin.consultech.de) as long as this information wasn't confidential or did not hinder the process of intellectual property protection. The EUROCALIN website was visited on average about 82 times each month while 34% of the visitors continued exploring the site. 50% of the visitors were accessing the site from Europe, 24% from USA/Canada and 13% from Asia.

Furthermore, flyers were generated and spread at conferences among the visitors of our booths or by the partners. We consider our presentations were very important for the consortium in terms of visibility to and feedback from the community.

The project and its results have been presented in specific conferences across Europe, North America and Asia. These activities were the starting point for possibilities to exploit the results of the project. With up to more than 30,000 visitors each, the scientific conferences attended were the largest worldwide and represented the ideal platform to inform a broad scientific community about the project and make it aware at the same time of the importance of Anticalins and their therapeutic applications.

American Society of Hematology, Annual Meeting 2011	(20,000+ visitors)
American Association for Cancer Research (AACR) Annual Meeting 2012	(17,000+ visitors)
American Association of Clinical Chemistry 2012	(20,000+ visitors)
American Society of Nephrology 2012	(13,000+ visitors)
American Society of Clinical Oncology (ASCO) Annual Meeting 2013	(30,000+ visitors)
BIO International Convention 2015	(15,858 visitors)
American Society of Nephrology (ASN) meeting 2015	(13,000+ visitors)
American Society of Hematology (ASH) Annual Meeting 2015	(20,000+ visitors)

Journalists accredited at these conferences ensure a broad dissemination across medical specialisations, countries and continents. In addition, scientists of the consortium presented the project's aims, results and progress. The podium presentation at ASH 2015 on the results of the project and the first in man clinical trial was very well attended by about 300 people.

Two peer reviewed publications were published from the collaboration between Radboud University Nijmegen Medical Centre (RUNMC) and Pieris:

Laarakkers et al., Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform, PLoS One 8(10):e75518, 2013

Grebentchikov et al., Engineered Human Lipocalin as an Antibody Mimetic: Application to Analysis of the Small Peptide Hormone Hepcidin, Clinical Chemistry 60(6) 897-9, 2014

Four more publications are in preparation at the partners and will be published soon.

Work of the consortium has led to 3 patent applications which are leading into further lines of development into products.

Patent PCT/EP2011/064086 "Binding Proteins for Hepcidin"

Patent PCT/EP2012/075135 "Methods for preventing or treating disorders by increasing bioavailability of iron and related pharmaceutical formulation"

PCT application of “Novel Lipocalin-Mutein Assays for Measuring Hepcidin Concentration” PCT/EP2014/052228 (WO2014122166)

A multitude of discussions and interviews with various stake holders important for the future exploitation of the project were realised by the consortium´s partners. The scientists and physicians covered different kinds of medical disciplines:

- Experts in hepcidin biology: to discuss effects of hepcidin inhibition and nonclinical testing
- Nephrologists: to discuss CKD patient populations, medical need, existing therapies and target product profile
- Oncologists / haematologists: to discuss oncology patient populations and potential indications, medical need, existing therapies
- Clinical development experts: to discuss the clinical development and regulatory strategies.

The consortium participated at several business-to-business (B2B) conferences attended by companies active in pharmaceutical and clinical development. Therefore, partners presented actively at business conferences in the field of general business development such as the "BioEurope", "Bio International Convention", "J.P. Morgan Annual Healthcare Meeting" and more subject-specific conferences such as the "Next Generation Protein Therapeutics" meeting, the "Antibody Engineering" meeting, the "PEGS Protein & Antibody Engineering Summit", the "Recombinant Antibodies" meeting and the "International BioIron society" meeting. The aim was to be visible to companies interested in using the Anticalin technology in clinical development or hepcidin assay technology in the diagnostic field. This led to a number of fruitful discussions between the consortium and interested companies, some of which

are still being pursued in order to co-develop some of the technologies developed in the course of EUROCALIN.

To exploit the results of the EUROCALIN project, the dissemination activities especially at pharma and biotech partnering events as well as at the scientific and business conferences were extremely useful. Valuable contacts with large international pharmaceutical companies were established for further strategic steps, including licensing of the project and related rights. In addition, regulatory experts, clinical development experts as well as specific clinicians have been contacted to advance the project´s development and its results beyond the project´s duration.

4.1.6 Project public website and contact details

The project's logo

The project's website is available under eurocalin.consultech.de
Here, flyers and posters as well as contact details of the partners and the coordinator are found.

Contact details of the coordinator:

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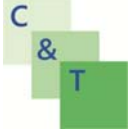
	Participant legal name	Website
	Pieris Pharmaceuticals GmbH (former Pieris AG)	www.pieris.com
	Technical University of Munich	www.tum.de
	Medical University of Innsbruck	www.i-med.ac.at
	Radboud University Nijmegen Medical Centre	www.ru.nl
	Covance Laboratories Ltd	www.convance.com
	Antitope Ltd	www.antitope.co.uk
	Fujifilm Diosynth Biotechnologies Ltd.	www.fujifilmdiosynth.com
	Coriolis Pharma Research GmbH	www.coriolis-pharma.com
	FGK Clinical Research GmbH	www.fgk-cro.com
	ConsulTech GmbH	www.consultech.de