Translating disease to cardiovascular health
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Publishable summary

TransCard is a collaborative effort between two industrial and four academic partners from four European countries, i.e. Switzerland (UZH and NEBION; lead small medium enterprise), Germany (PolyQuant; second small medium enterprise), Denmark (REGIONH) and the Netherlands (UMCG, AMC). This consortium aims at identifying and characterizing novel factors that are associated with blood lipid levels which thereby affect the risk of developing atherosclerotic cardiovascular disease (CVD), the number one killer in the EU. Because the concentration of LDL cholesterol in blood is causally related to CVD, we focus on this lipid trait.

NEWLY IDENTIFIED TARGETS

Over the past 1.5 years, our consortium has experienced exciting times: through a whole genome wide siRNA screen, the UZH identified multiple targets that affect the uptake of LDL and/or HDL in hepatoma cell lines. To validate the involvement of these novel targets, several routes have since been employed. These include studies in mice in which target genes were downregulated using CRISPR/Cas9 (UMCG) while REGIONH has conducted exploratory studies whether variation in these genes affects plasma lipids and ischaemic disease. In addition, the target gene lists were prioritized (UZH/UMCG/NEBION) and a dedicated gene panel was developed to sequence all genes that were identified through TransCard efforts in several target populations. These include individuals with extreme LDL cholesterol levels who were selected by the UMCG from the general population in the Netherlands (www.lifelines.nl; n=167.000) as well as patients with familial hypercholesterolemia of unknown origin (AMC and others).

UPDATE ON OTHER TARGETS

The choice to further unravel the mechanisms by which *COMMD1* affects plasma cholesterol by the UMCG has proven very valuable for TransCard. Following the initial finding that COMMD1 affects the trafficking of the low-density lipoprotein (LDLR) to the membrane of hepatocytes, which in turn controls the levels of circulating LDL cholesterol, we have now provided evidence that COMMD1 acts in concert with other members of the CCC complex as well as additional protein complexes (WASH/Retromer). The function of these protein complexes in endosomal cargo (such as LDLR) sorting, is evolutionary conserved and is shown to control plasma cholesterol concentration in mice, dogs and humans. In P3, we have made a second important step in translating this new basic insight to relevance for human disease in that our data show that variation in one of the genes affects levels of LDL cholesterol in plasma and risk of atherosclerosis in the general population. We have furthermore finalized our studies on *LRP1* as long-established candidate gene but for which no evidence existed in the public domain whether it has a role in human lipoprotein biology. In a recent publication, we provide the first evidence that rare LRP1 mutations affect HDL metabolism. *STAP1* (identified by the AMC) is still under study as candidate gene for familial hypercholesterolemia. The gene is primarily expressed in cells of the immune system and could connect the lipid and immune research fields. This could be of great value when considering the natural aetiology of atherosclerosis. In this light, bone marrow transplantation studies in mice and studies in carriers of *STAP1* mutations are planned and ongoing, respectively. TransCard's lead small medium enterprise, NEBION, has identified multiple novel candidate genes through *in silico* analyses. Two of these genes are at the heart of ongoing *in vitro* and in experimental animal studies. While one of these genes may be targetable with small molecular inhibitors, the other gene may be well-suited for liver-directed anti-sense therapy. After a successful drug screen for genes that control the transport of lipoprotein across endothelial cells by UZH has finally continued its studies on genes that control the transendothelial transport of lipoproteins, a key targetable event in atherogenesis.

TECHNICAL ADVANCES

NEBION is continuously gaining power to identify new genes after curating additional publicly available mRNA datasets and is developing tools to prioritize genes for further study. In addition, the use of CRISPR/Cas9 to quickly study the impact of liver specific ablation of novel candidate genes has been and is

fully exploited. Further support comes from targeted proteomics studies, enabled by PolyQuant (QconCAT technology) and mass spectrometry support from the UMCG, which provides insight into quantitative changes in multiple proteins involved in lipid and lipoprotein homeostasis.

CONCLUSION AND OUTLOOK

TransCard has in addition to several 'stand alone genes' identified three series of novel genes with different roles in lipid and lipoprotein metabolism. These include genes involved in the intracellular trafficking of major lipoprotein receptors but also genes involved in unanticipated biological phenomena. The ongoing work is aimed at improving our insight into the molecular aetiology of the genotype-phenotype relations and to discern which genes and their products could potentially be targeted with pharmaceutical strategies. In addition, UZH has revealed that the transendothelial transport of lipoproteins is an interesting target for therapeutic intervention in atherosclerotic CVD. It is unfortunate, however, that TransCard is soon coming to an end (September 2018) which will not enable us to continue the work that was started. Naturally, our team is actively seeking for continuous support from the European Commission to *'harvest what was seeded'*.

1.1. Description of project context and objectives

TransCard has set out to identify and characterize (novel) genes that are associated with blood lipid levels and thereby affect the risk of developing cardiovascular disease (CVD), the number one killer in the EU. Because the concentration of LDL cholesterol in blood is causally related to CVD, we focus on this lipid trait. The patient who suffers from a severe lipid disorder is closest to the core of the TransCard efforts. The rationale for this choice is straight forward: when finding the origin of human disease, there is no need to translate findings from an experimental animal model or a tissue culture dish to human biology. However, finding novel origins of rare human lipid and lipoprotein disorders can be a long, risky and bumpy road and we therefore also employed several other routes of target identification through *in silico, in vitro*, and animal studies. The consortium expertise includes human validation of novel targets through genetic epidemiology studies prior to or alongside experiments that focus unravelling the underlying molecular mechanisms how genes are related to the phenotype. The latter basic studies will be of key importance to advance our understanding of how to intervene in metabolism to best fight CVD.

1.2. Descriptions of work performed and main results

NEWLY IDENTIFED CANDIDATE GENES

One of the major achievements in the last period was the finalization and validation of the hits of an image-based RNA interference screening for genes that limit the uptake of HDL and LDL by hepatocytes by UZH. The RNAi-screen of HDL and LDL uptake by hepatocytes revealed 152 genes regulating HDL uptake and 83 genes regulating LDL uptake. Replication experiments of 20 top hits confirmed their limiting effect on LDL uptake but not on HDL uptake. Therefore, the subsequent functional validation *in vitro*, mice and humans has been focused on LDL uptake. Most of the genes limiting LDL uptake were clustered into four functional groups of which two appeared the most interesting.

By combining knock-down with RNA sequencing, a first group of genes was identified that affect the LDL receptor pathway. This was validated in human liver and blood cells. The expression of one of the genes correlates with LDL cholesterol and age in blood cells (UMCG). Preliminary evidence indicates that the knock-down of two top candidate genes in experimental mouse models leads to an increase in LDL cholesterol (UMCG). In the population, some common genetic variants were found associated with LDL cholesterol (REGIONH). Several patients with severe (familial) hypercholesterolemia carry mutations in the respective genes and family studies are ongoing to show genotype-phenotype segregation (AMC/UMCG).

Knock-down of second set of genes limit the abundance of LDLR on the cell surface and LDL-uptake by hepatocytes. Variation in one of respective genes is associated with LDL cholesterol in the general

population and several patients with severe (familial) hypercholesterolemia carry mutations in this set of genes. As above family studies are ongoing to show genotype-phenotype segregation.

DEEP SEQUENCING OF ALL TRANSCARD CANDIDATE GENES

The top hits of the screen performed by the UZH described above have become part of a dedicated next generation sequencing kit that is used by the UMCG to study all TransCard candidate genes. This gene panel performs very well (extremely high coverage and sequence depth) and is used to sequence patients and individuals with unexplained high or low LDL cholesterol. The genomic DNA of >400 individuals have now been sequenced and we have started the first segregation analyses of rare genetic variants in key candidate genes in families with unresolved familial hypercholesterolemia.

PROGRESS ON CANDIDATE GENES IDENTIFIED IN P1 AND P2

The TransCard consortium has naturally continued its studies into candidate genes that were identified in earlier stages of the project:

Starting with liver-specific *Commd1-/-* mice in the first year, we have also studied the involvement of multiple associated genes, e.g. *COMMD6*, *COMMD9 and CCDC22* in the cellular trafficking of the receptor for LDL (LDLR). We have collected accumulating evidence that not only LDLR but also the trafficking of other lipoprotein receptors is affected when members of associated protein complexes are downregulated. While rare disorders already helped to validate the role of some of these genes in the regulation of plasma lipid levels in humans, we have with the help of our partners in REGIONH been able to take this to a next level: common variation in one of our candidate genes is associated with plasma lipid levels as well as risk of myocardial infarction in the general population.

In the first year, an interesting candidate was identified through *in silico* analyses by NEBION as being strongly coregulated with lipoprotein lipase, a key enzyme in plasma triglyceride hydrolysis. While our initial studies in mice were very promising, further studies have been disappointing: the whole-body knockout mice initially showed lower plasma lipids and reduced body weight gain but this phenotype was lost when crossing the mice to a C57Bl/6 background which is needed for atherosclerosis studies. In addition, initial population studies by REGIONH showed an impact on LDL cholesterol and apolipoprotein (apo) B but further studies also showed effects on both HDL cholesterol and apoAI and combined these results did not show an effect on ischaemic disease. Ongoing *in vitro* studies, however, still show promise as downregulation of this gene increases the membrane expression of key lipoprotein receptors such as LDLR and LRP1 while overexpression of the gene causes downregulation of these key proteins. We therefore continue our studies into the how a factor that must be present in cell culture media can have such an important impact on lipoprotein receptor expression.

STAP1 was initially identified by the AMC as a candidate gene for familial hypercholesterolemia in patients but the gene is primarily expressed in immune cells. *Stap1-/-* mice, generated by the UMCG, however showed no effect on plasma lipids. In addition, REGIONH could not identify adequate tools for genetic analyses to validate a role for *STAP1* in lipid metabolism. In a final step, the UMCG will perform bone marrow transplantation studies to study the impact of a loss of haematopoietic *Stap1* expression on atherosclerosis. In addition, the AMC is studying peripheral blood cells of patients with *STAP1* mutations and family controls.

A second gene identified by NEBION is still under investigation. The UMCG found out that homozygous loss of this gene is lethal. Heterozygous mice, however, show decreased fasting triglycerides on a chow diet. Remarkably, REGIONH has not found a single gene variant when sequencing 200 individuals with the highest or lowest LDL cholesterol levels. The UMCG therefore increased their efforts to find human variation as a start for further studies (using the dedicated gene panel that is described above). These studies will be directed to subjects with extreme plasma triglyceride phenotypes.

Another gene was identified through literature search by UMCG. The encoded enzyme plays a role in the *de novo* cholesterol synthesis. The UMCG generated whole body knock-out mice which did not show a plasma lipid phenotype. Interestingly, however, a high-fat diet induced a strong downregulation of this gene in wild-type mice. The mice are being bred to a C57Bl/6 background for further studies and caloric restriction experiments are planned.

VEGF and sphingosine-1-phosphate (S1P) have enjoyed continuous interest by UZH as regulators transcytosis and retroencytosis of lipoproteins in endothelial cells. SR-BI mediated intracellular accumulation of intact lipoproteins was identified as the likely origin of cholesterol accumulation and the characteristic clear cytoplasm of clear cell renal cell carcinoma (ccRCC). VEGF induced SR-BI cell surface translocation may be the underlying mechanism and the resulting enhanced SR-BI/lipoprotein interaction may contribute to proliferation and hence prognosis of ccRCC. The immunochemical analysis of kidney carcinomas revealed significant associations of the abundance of apoA-I and apoB but not SR-BI with subtype, tumor stage, differentiation grade, and markers of HIF-1α activity as well as survival.

S1P and its cognate receptors S1P1 and S1P3 regulate the transendothelial transport of HDL and LDL in an antagonistic manner: LDL transport through endothelial cells was promoted by inhibitors or siRNAs of S1P1 and S1P3 via fluid phase (S1P1) or an as yet unresolved mechanism (S1P3). Conversely, HDL transport was promoted by agonists of S1P1 and S1P3 by an SR-BI dependent mechanism. These findings support the concept that transendothelial transport of lipoproteins happens through an active and regulated process rather than by passive filtration and thus make this process an interesting target for therapeutic intervention in atherosclerotic CVD.

Finally, NEBION's tools to identify and prioritize genes of interest have been improved and are still being used by partners. Our second SME, Polyquant, has and is providing continuous help to TransCard through the production of QconCATs to study proteins of interest in serum, plasma as well as tissue homogenates. This has enabled us to study e.g. the effects of specific hepatic depletion of candidate genes on established and new players in lipid homeostasis. This has been of special great value for our studies into the trafficking of major lipoprotein receptors. We have also tested the use of QconCATs for the simultaneous detection of proteins with major roles in lipid and lipoprotein metabolism in plasma of individuals with extreme LDL cholesterol levels (UMCG). These results have helped us to design novel QconCATs for the detection of low, medium and high abundant proteins to help our investigations into the risk of developing CVD.

1.3. Expected final results and potential impact and use

We expect that our studies in mice will continue to help our basic understanding of endosomal trafficking of major lipoproteins to the cell membrane. Since the presence of the reappearance of these receptors at the cell membrane has a direct impact on circulating levels of LDL and other lipoproteins, it may provide interesting insights for therapeutic intervention. However, a complete loss of most of these factors is lethal in mice. On the other hand, there are already compounds that stabilize one of the involved proteins. Our studies moreover show that mutations in one of the genes studied can decrease plasma LDL cholesterol and concomitantly the risk of myocardial infarction. This clearly underlines the importance of this study field for human lipid metabolism and atherosclerosis which is increasingly being recognized in the field. Our ongoing targeted sequencing efforts using dedicated TransCard gene panels in patients with unresolved familial hypercholesterolemia have identified multiple rare variants in the genes that encode for the genes in the respective protein complexes. This will help to boost our segregation studies in affected families.

The first gene that we identified with NEBION remains an interesting gene for further study after our recent discovery that a loss of expression increases the presence of major lipoprotein receptors (LDLR and LRP1) at the cell membrane. Blocking the action of this receptor holds promise to reduce plasma lipid levels and decrease body weight gain. We will seek in this regard collaboration with experts on the production of synthetic compounds to blunt its activity. From the other genes studied over the last few years, we will specifically push our studies into the second gene that was identified through in silico analysis. This because it is strongly coregulated with lipid gene in men and mice which is already a target for promising therapies.

Second, because it is exclusively expressed in the small intestine and liver which would in theory reduce chances of unwanted side effects when the gene is targeted in e.g. the liver. We will thus continue our studies into the mechanisms that reduce plasma triglycerides in our mouse model. This in itself is remarkable when considering that mice are very fast triglyceride metabolizers. Since there are no evidence-based drugs that lower plasma triglycerides and risk of atherosclerosis, this project holds promise for therapy beyond cholesterol-lowering with statins and PCSK9 inhibitors.

As indicated, P3 has been an exciting time with the discovery of many novel targets by UZH through a genome wide screen for genes that affect hepatic lipoprotein uptake. TransCard will conclude segregation studies on hypercholesterolemic patients and their families with mutations in the sets of genes that were identified. Segregation of mutations with hypercholesterolemia will lead to the unravelling of novel genetic causes of familial hypercholesterolemia. In addition, it will be important to understand the mechanisms that regulate LDL receptor activity because this may unravel targets for the development of therapeutic interventions.

The discoveries of lipoprotein accumulation in ccRCC by UZH may finally be exploited for risk stratification and drug development. In addition, UZH will conclude the work on the role of S1P receptors for the regulation of transendothelial lipoprotein transport by investigating proteoglycans as the target, by which S1P3 inhibition promotes the transendothelial transport of LDL and by testing the effect of endothelial S1P1 and S1P3 knock-ins on transendothelial lipoprotein transport. UZH expects that the antagonistic regulation of transendothelial HDL and LDL transport by S1P receptors will attract a lot of attention by academia and industry: it will serve as the current strongest evidence that the permeation of the endothelium by lipoproteins is specific and can be targeted for the treatment of atherosclerosis as well as for the delivery of drugs and imaging tracers into many organs including the arterial wall.