IMI1 Final Project Report Public Summary

Project Acronym: MARCAR

Project Title: bioMARkers and molecular tumor classification for non-genotoxic CARcinogenesis

Grant Agreement: 115001
Project Duration: 01/01/2010 - 30/06/2015

Executive summary

The executive summary will be made publically available, and therefore should not include information deemed as confidential by the consortium. It should be concise (preferably no more than 40 pages), comprehensive and should capture the updates for the last reporting period as well as the overall outputs of the project and its impact. It shall at least cover the following items:

1.1. Project rationale and overall objectives of the project

Overall aim:
Current methods used in drug development lead to a large number of drugs not reaching the market due to the late discovery of cancerous effects during pre-clinical trials. Such cancers are not the result of direct genetic toxicity, as drugs that exhibit such characteristics are eliminated from consideration early in their development. They can however result from changes in gene expression or cellular phenotypes caused by mechanisms other than changes in the underlying DNA sequence. Drugs that induce such cancers are called non-genotoxic carcinogens (NGC). At present due to a lack of validated short-term study techniques such non-genotoxic cancer causing drugs tend to be identified following prolonged pre-clinical studies. Using the liver, the major target organ for such drug-induced tumours, the MARCAR project aims to advance our understanding of NGC mechanisms and the availability of early "biomarkers" of NGC action to help in the design of more predictive short term assessment tools to reduce the requirement for costly long-term biological testing including reduction of animal numbers and help to deliver safer and faster medicines to patients.

The main objectives of the project were to:

- Identify early biomarkers for more reliably predicting which compounds have a potential for later cancer development
- Improve the scientific basis (mechanisms and translatability) for assessing carcinogenic potential of non-genotoxic drugs
- Identify the molecular response to NGC exposure that underpins development of 'early exposure' biomarkers
- Improve drug safety and efficiency of drug development, and progress the development of alternative research methods (such as the "3Rs" concept: i.e. reduction, refinement and replacement of animal experimentation).

These will ultimately reduce or eliminate the requirement for costly long-term biological testing and allow drugs to be developed with more confidence of success.

1.2. Overall deliverables of the project

MARCAR aimed to achieve the above objectives by implementing techniques that, at a molecular level, can identify changes occurring as a result of exposure to NGC compounds. This analysis aimed to predict the induction of cancer in well-developed pre-clinical models similar to those currently used by pharmaceutical companies for identifying non-genotoxic carcinogens.
State-of-the-art analytical techniques were used to identify the cause of precancerous changes and tumours that develop as a result of exposure to NGC. Understanding the mechanism of action of the NGCs was an important step in identifying candidate biomarkers for further analysis by the MARCAR project. To analyse the validity of the biomarkers the results from these studies have been collated using novel bioinformatics approaches and standardised data management. The output will help to identify novel candidate biomarkers that can be used to identify the causes of cancer development after exposure to NGC.

One of the main drivers behind finding early biomarkers for risk assessment is the potential to reduce animal use in drug and chemical risk assessment, i.e. the NC3Rs directive on the reduction/refinement/replacement of experimental animal use. MARCAR aims to tackle this issue by developing methods for conducting reliable short-term studies which will reduce the need for long-term biological testing.

1.3. Summary of progress versus plan since last period

(Any major deviations, risks should be highlighted in this section)

During this period MARCAR has focussed on completing the remaining objectives of the project. It became clear at the beginning of the period that there were factors which made it desirable to extend the duration of the project by 6 months. These factors were:

1. Bioinformatic Analysis - The time required to complete de novo 28 day rodent studies covering a range of distinct non-genotoxic carcinogen modes of action and the resultant molecular profiling of tissue samples generated by MARCAR exceeded the initial expectations. An extension to M66 was requested to enable extensive bioinformatics data analysis of omics datasets generated by the partners. The in-depth biological interpretation of the processed data produced by EKUT-B was subsequently performed by the partners involved in data generation (EKUT-A, BSP, UCB, LUB, NOV, UNIVDUN, UEDIN, MUW and INSERM).

2. Validation of MARCAR biomarkers – The identification and refinement of the MARCAR biomarker list took longer than expected (dependent in part on the bioinformatics analysis outlined above). Additional time was therefore also requested to complete quantitative and qualitative evaluation of a subset of biomarkers on MARCAR samples generated in the confirmatory phase. This allowed an assessment of biomarker specificity for distinct non-genotoxic carcinogen modes of action and potential human relevance (e.g. comparison of rodent liver vs. humanised mouse models vs. human liver-derived cell models) to be performed.

While we have not clearly identified biomarkers which have enhanced relevance to human risk assessment, we have generated significant amounts of data across several platforms. We remain confident that, together with other members of the MARCAR consortium, we will be able to create a list of potential biomarkers for application to risk assessment for non-genotoxic carcinogenesis in humans. However, given the complexity and volume of data, it will undoubtedly require extensive analysis beyond the scope and timeframe of the MARCAR project. Furthermore, since the data will become publicly available, interested researchers may then extract further insight by asking specific research questions or performing specific analyses.

The data obtained in tasks 3.3., 3.4., and 3.5 clearly show that (i) two prototypical NGCs act in a different way within one species (rat) and that (ii) one NGC elicited effects differing between rat and mouse liver. Thus, the development/validation of a set of biomarkers predicting the action of different NGC in different species appears to be a greater challenge than anticipated.

1.4. Significant achievements since last report

MARCAR has continued to progress in this period. Significant results include:

- Identification of mRNA signatures which indicate the initiation of cancer. These biomarker candidates improve the understanding of the effects NGC have on the liver and how they may induce cancer.
- Identification of species (rat vs. mouse)-specific miRNA changes in the liver in response to NGC treatment

- Establishing that a mechanism of drug metabolism, previously shown to be important in the rodent liver, is also active in cultured human liver cells. These cells therefore represent a powerful tool to evaluate toxicity of drugs directly in human cells.
- Understanding the role of the mesenchymal cells (a particular type of liver cell) in NGC-driven liver cancer.
- Identification of tumour-specific alterations in cell metabolism. Knowledge about metabolic reprogramming in tumor cells is of potential interest for diagnostic purposes and the development of tumor biomarkers and may also lead to previously unexpected cancer treatment strategies.
- Publication of a web tool (ToxDBScan) for quick and easy evaluation of the ability of drug candidates to cause cancer. ToxDBScan screens new drug candidates against two large-scale public databases, which contain expression profiles for substances with known cancer-causing profiles.
- Updating InCroMAP, a tool originally developed to analyse genomic and proteomic data. In the extended version analysis of metabolomics data is also included thereby creating a tool capable of evaluating more comprehensive systems biology studies.
- Establishing that phenobarbital can induce cell cycle transcriptional responses and can promote liver tumours via interactions with nuclear receptors.
- Development of a tool (RPPApipe) to process and analyse reverse-phase protein arrays (RPPA). Previously available analysis pipelines had only limited support for RPPA, thus RPPApipe was developed specifically to provide RPPA analysis thus complementing the existing repertoire of microarray analysis tools.
- Description of two novel methodologies for the reproducible extraction of characteristic mRNA signatures, which were employed to capture specific gene expression changes observed for nongenotoxic carcinogens.
- Establishing that the adaptation of mammalian cells to culture systems involves early epigenetic and transcriptional reprogramming. These effects occur much earlier than previously thought and have significant implications for the use of cell lines as reliable models.
- Publication of a model of oxidative stress, showing marked differences in the ability of nongenotoxic carcinogens to induce oxidative stress. This suggests that oxidative stress is not a key determinant of nongenotoxic carcinogenesis, but may contribute in a drug-specific manner.
- Establishing that superoxide may contribute to the promotion of liver cancer.
- Publication of a review focussing on how recent advances in the field of epigenetics can enhance our understanding of drug exposure and provide novel safety biomarkers.
- Development of DNase-seq methodology for mapping of phenobarbital-induced modulation of the liver cistrome and generation of novel insights into the molecular basis for species-specific differences in NGC mechanisms.

1.5. Scientific and technical results/foregrounds of the project

The main results of the project are as follows:

1) Optimization of tools for measurement of new biomarkers in tissue and blood, including micro-RNAs.

    - Development of miniaturised assay systems that are capable of semi-quantitatively detecting more than 200 regulatory proteins (total and activated forms).

- Generation of a novel transgenic reporter model which utilises the haem oxygenase-1 promotor to drive LacZ expression in order to measure oxidative stress induced by a range of non-genotoxic carcinogens (NGCs) Almost all of the NGC's tested induced oxidative stress but to differing extents. We are currently relating the extent of oxidative stress to other cellular changes, ie gene expression and methylation status

- Generation of a novel reporter model, where the glutamine synthase gene, known to be induced during NGC carcinogenesis, has been linked to multiple reporters, including those which allow in vivo real time luminescent, MR and PET imaging.

- Development of Magnetic Resonance Imaging (MRI) protocols which allow detection of tumour lesions at a size of 1 mm. This allows for non-invasive tracking of tumour progression and therapeutic responses in small animal models. Thus, the established methods are extremely valuable for the MARCAR consortium to assess tumour burden. Currently we are working on new biomarkers for non-invasive imaging of liver tumours using PET in combination with MRI.

2) Further understanding of the genetic and epigenetic effects of NGC.

- Demonstration of the relationship between dose dependent changes arising at the 5hmc mark following drug exposure and changes in the mechanism of epigenetic regulation prior to the appearance of late stage cancer morphologies

- Demonstration that changes at the highly dynamic 5hmC mark follow transcriptional changes in response to PB exposure and can accurately discriminate drug exposure based on the length of dosing.

- Demonstration that the phenotype of a liver tumour in model systems is mainly driven by the genetic alteration driving tumourigenesis, and that NGCs select for a NGC class-specific geno/phenotype.

- Identification of mechanisms which can be used to understand the effects of NGC on at a metabolic level.

- Demonstration of the decisive role of the CAR nuclear receptor and Wnt signalling pathway in regulation of the non-coding RNA Gtl2/Meg3, a novel potential biomarker for NGCs that may represent reprogramming or de-differentiation to a stem cell-like state.

- Demonstration that primary human liver cells in co-culture represent a valuable in vitro system to characterise the effect of long-term exposure to prototypical compounds. Next steps will be to identify a specific gene signature for non-genotoxic carcinogenesis (NGC), compared to genotoxic carcinogen (GC) and non-carcinogen compounds (NC) in this model.

- Demonstration of gene locus- and species-specific changes in the liver cistrome in response to PB exposure

3) Development of new bioinformatic tools for interpretation of study outcomes

- Development of a database of study results which correlates the traditional toxicity endpoints in terms of clinical chemistry and histology with new biomarkers

- Development of a tool which facilitates the pathway-based analysis and visualization of heterogeneous cross-omics datasets, InCroMAP (http://www.cogsys.cs.uni-tuebingen.de/software/InCroMAP). The tool is extensively used by the MARCAR academic and industry partners and has been developed and published within the framework of the MARCAR project.

The project has generated the following publications:

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1.6. Potential impact and main dissemination activities and exploitation of results

Please explain how the project scientific/technical outputs contribute to the overall IMI objectives:

- *to provide socio-economic benefits for European citizens,*
- *to contribute to the health of European citizens,*
- *to increase the competitiveness of Europe and help to establish Europe as the most attractive place for biopharmaceutical research and development.*

Please outline how the project outputs have/will have the potential to be rapidly and broadly spread and taken up within the scientific/industrial community and healthcare professionals.

A good understanding of the effect of a drug at the molecular level will ensure a better safety assessment and lead to safer drugs with less adverse effects. In addition, knowledge about the mechanisms underlying carcinogenesis may also help in treating cancer patients. Better understanding of the associated pathways that respond to drug exposure offers the promise of new therapeutics that bypass these steps. If this can be achieved, it will represent significant savings, both

in time and costs, in the drug development process and be of considerable benefit to patients in terms of drug safety.

A number of recent publications analysing epigenomic parameters suggest that changes in the epigenome can represent necessary conditions to enable or at least enhance changes in the activity of genes and thus the resulting phenotype. The MARCAR project has already demonstrated that it is possible to detect physiologically relevant changes in response to drug exposure. Ultimately, it is expected that the data generated by MARCAR will form the basis of an NGC 'signature', which will allow us to predict whether a particular drug or chemical is likely to be a non-genotoxic carcinogen. Such scientific output from MARCAR would result in a significant reduction in the use of animals (rats, mice), and a refinement of the testing process such that the current rodent 2 year bioassay may be replaced by a combination of molecular and phenotypic endpoints in sub-chronic toxicology studies.

In addition, MARCAR is also exploring the possibility of reducing animal use through our exploration of the potential for imaging to identify biomarkers. The project has already demonstrated the ability to identify liver tumours in rodents by magnetic resonance imaging and to follow their development over time non-invasively. This capability could potentially reduce the number of animals used in carcinogenicity screens and allow the analysis of chemotherapeutic intervention in significantly reduced animal cohorts.

Certain analytical techniques and sampling procedures have been implemented into the early phase of the drug development programme at partner sites.

Predicting non-genotoxic carcinogenesis in preclinical development is a major challenge for the development of drugs intended for chronic administration in humans. The identification of early NGC mechanisms and biomarkers will provide industry and regulatory scientists with new tools for earlier decision-making, mitigation of positive carcinogenicity findings and cancer risk assessment.

The MARCAR project has been contributing to discussion on the enhancement of preclinical carcinogenicity testing strategies in the context of recently proposed changes to the ICH S1 guidance on Rodent Carcinogenicity Testing of Pharmaceuticals (Regulatory Notice Document 8th August 2013). The goal of this potential change is to introduce a more comprehensive and integrated approach to address the risk of human carcinogenicity of small molecule pharmaceuticals, and to define conditions under which 2-yr rat carcinogenicity studies add value to that assessment. The ICH revision proposal envisions that Sponsors of pharmaceuticals would provide Drug Regulatory Agencies a Carcinogenicity Assessment Document (CAD) which could justify a 'waiver request' that seeks to omit the conduct of 2-yr rat carcinogenicity studies. Weight-of-Evidence Factors for consideration in a CAD include knowledge of intended drug target and pathway pharmacology, secondary pharmacology, & drug target distribution in rats and humans. In particular, it is anticipated that new biomarkers, emerging technologies, and alternative test systems (e.g. such as innovative mechanism-based biomarkers and/or models developed by MARCAR) will be submitted with scientific rationale to help explain or predict animal and/or human carcinogenic pathways and mechanisms.

1.7. Lessons learned and further opportunities for research

Please indicate how the collaboration in a public private partnership (PPP) has been an added value to achieve the objectives of the project.
From your experience, please propose any recommendations/solutions which could be useful for a PPP.
In view of your project achievements, please provide your views on potential new research to further advance the field.

The collaboration in a public private partnership enabled valuable discussion and knowledge exchange between industry and academic partners and has supported the different phases of the MARCAR project (animal study design, animal study performance and the evaluation of data). Collaboration between the EFPIA and academic partners has been instrumental in the development of new tools for the identification and validation of candidate biomarkers. The imaging component of the project at EKUT-C has benefited significantly from collaboration with the consortium partners. By drawing on the expertise and resources made available by the partners they have been given access to relevant animal models and identified key-biomarkers for liver tumour detection which will be transferred into Positron Emission Tomography (PET) imaging probes which we hope can be used to non-invasively identify early biomarkers.

Another significant impact of the collaboration between the EFPIA and academic partners has been the development and implementation of processes, across the project, which meet the rigorous standards required by Pharma. The tissue samples and resulting data generated by the project have been used to further our understanding of the mechanism of action of NGC's and has ultimately allowed the consortium to identify candidate biomarkers that are representative of the action of NGC.

The success in developing the novel bioinformatics tools that have been used to validate data derived from the MARCAR project can also be attributed to the fruitful collaboration between the academic and industrial partners. Evidence of this success can been seen in the recently published research article "A Toxicogenomic Approach for the Prediction of Murine Hepatocarcinogenesis Using Ensemble Feature Selection" in PLOS ONE, in which the results from analysis performed at the University of Tübingen, under the guidance of the EFPIA partners, were presented.