1. FINAL PUBLISHABLE SUMMARY REPORT

Essential metals like iron, copper and zinc are required by all organisms to thrive, however, the same properties that make these metals necessary for life make them also toxic. Therefore, organisms have evolved systems to handle metals, avoiding dangerous and wasteful nonspecific interactions. Several proteins are involved in the tight regulation of intracellular metal concentration that is defined as metal homeostasis. Understanding if metal based nanoparticles (NPs), now present in several consumer products, interact and affect the function of metal transporter proteins resulting in a disrupted cellular metal homeostasis was the main objective of the NanoTraffic project. As model system a fish intestinal-hepatic cell *in vitro* system from rainbow trout was developed (Fig. 1A). Influence of metal NPs on essential metal homeostasis was evaluated measuring intracellular metal concentrations along with transcriptional (copper transporters and metal detoxification genes) and post-translational (ATP7A protein trafficking) changes elicited in cells on exposure to metal-NPs.

The project was divided into three steps: 1) establish polarized *in vitro* preparations and assess cell viability; 2) quantify uptake and intracellular fate of metal-NPs; and 3) evaluate cellular responses to metal-NPs.

Step 1: The intestinal-hepatic cell culture system was developed. This system enables the evaluation of transport of NPs (or dissolved ions from the NPs) across the intestinal epithelia and the evaluation of effects that these have on the intestinal cells and liver cells growing underneath (Fig. 1A).

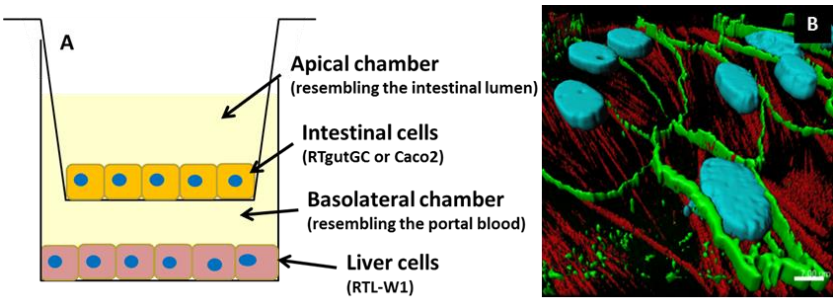

Figure 1. **A:** Schematic representation of the intestinal-hepatic cell system; **B**: Three dimensional view of RTgutGC stained for TJ (ZO-1; green), f-actin (Rhodamine Phalloidin; red) and nuclei (DAPI; blue). Image obtained combining several Z-stacks acquired by confocal microscopy followed by image analysis for 3D rendering.

Confocal microscopy analyses showed that RTgutGC grown on transwells for 3-4 weeks develop tight junctions and the cytoskeleton (actin) takes on a morphology typical of polarised epithelial cells (Fig. 1B,C). Moreover, RTgutGC cells develop a trans epithelial electrical resistance (TEER) comparable to the fish intestine *in vivo*. Other features of the fish intestine have also been shown in RTgutGC cells, including expression of Na/K- and V- ATPase at the mRNA and protein level and Villin at the mRNA level.

Step 2 & 3: The initial particle of choice was a citrate-coated silver NP (cit-AgNP; nominal size: 19 nm). Silver-based NPs are particularly well suited to test the effect of metal-NPs on copper homeostasis because ionic silver is known to be transported intracellularly by Cu transporter proteins (Bertinato et al., J Trace Elem Med Bio 2010, 24). In addition, two titanium dioxide NPs were selected as a non-dissolving NP. These were the commercially available Aeroxide P25 (P25-TiO_2NPs; size: 21 nm) and a fluorescently labelled TiO_2NPs, (Al-TiO_2NPs; size: 25 nm) (Blumenfeld et al., Inorganic Chemistry 2013, 52). The agglomeration behaviour of all NPs was analysed by dynamic light scattering (DLS) in growth medium (L15std; Leibovitz L15 supplemented or not with 5% Foetal Bovine Serum, FBS) and in exposure medium, L15ex, which is L15 without amino acids and vitamins. The agglomeration for all NPs was higher in L15ex than in the protein rich L15std, suggesting that proteins might have a role in steric stabilization of NPs agglomerates.

Cell viability and effective concentrations causing 50 % effects (EC50) have been determined in cells using a multiple endpoint assay (Schirmer et al., Toxicology 1998, 127). Three parameters of cell viability are measured simultaneously on the same set of cells: metabolic activity, membrane and lysosomes integrity. The cytotoxicity of the cit-AgNPs or $AgNO_3$ increased greatly in L15ex in which amino acids and proteins are removed. Remarkably, the endpoint most affected for cit-AgNPs was lysosome integrity (Neutral Red). This result led to the hypothesis that lysosomes are a key target of cit-AgNPs in these cells. This hypothesis was

confirmed by two additional experiments: (1) using a lysosome specific molecular probe, LysoTracker, an identical concentration response was obtained as for Neutral Red and (2) by Scanning electron microscopy (SEM) where cit-AgNPs could be visualised inside lysosomes (Fig. 2C). Elemental analyses by Energy-dispersive X-ray spectroscopy (EDX) confirmed that electron dense particles contained silver (not shown). Both of the TiO_2-NPs were not toxic at all doses tested (up to 1.25 mM). However, internalization of the NPs could be shown directly by SEM for P25-TiO_2NPs (Fig. 2D) and indirectly by measuring fluorescence in cells exposed to Al-TiO_2NPs (not shown). Differently from cit-AgNPs, P25-TiO_2NPs accumulated more in endosomes than in lysosomes (Fig. 2C,D).

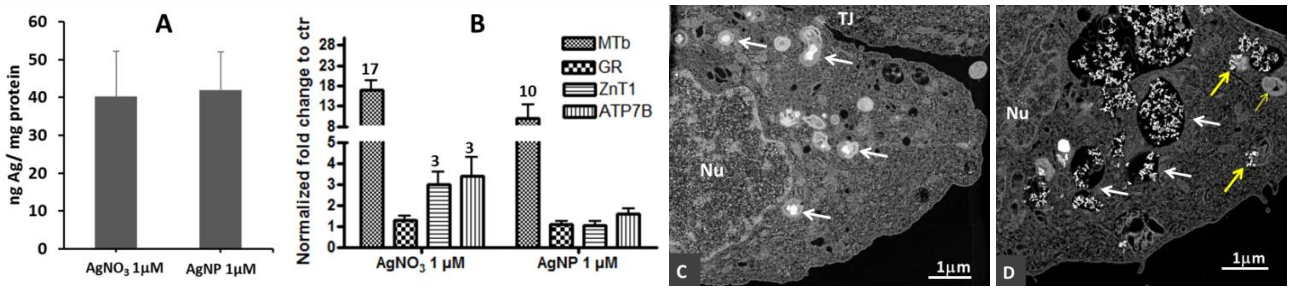

Figure 2. **A**: Accumulation of silver in RTgutGC cells exposed to 1 µM Ag as $AgNO_3$ or AgNP in L15ex for 24 hours. Values are means ± S.D. N=4. **B:** Transcriptional responses of Metallothionein B (MTb), Glutathione reductase (GR), Cu-ATPases (ATP7B) and Zinc transporter 1 (ZnT1). Target genes copy numbers were normalized to reference genes, EF1α and Ubiquitin. Bars bearing numbers are significantly different (means ± S.D. N=3, $P<0.05$; ANOVA, Tukeys test). **C:** SEM image of RTgutGC cells exposed to 50 µM cit-AgNP for 3 hours in L15ex. Arrows show electron dense particles in lysosomes. **D:** SEM image of RTgutGC exposed to 125 µM TiO_2 as P25-TiO_2-NPs for 24 hours in L15ex. White and yellow arrows show NPs in endosomes and lysosomes respectively.

The intestinal-hepatic cell culture system (Fig. 1A) has been exploited to evaluate transport of silver NP or silver ions across RTgutGC cells. Using the viability assays described above it was determined that applying apically 1 µM Ag as $AgNO_3$ or cit-AgNP for 24 hours was non-toxic to the RTgutGC cells while applying 3.4 µM $AgNO_3$ or 10 µM cit-AgNP resulted in a 15% reduction in viability (not shown). After exposure, the accumulation of Ag was measured by ICP-MS in RTgutGC and RTL-W1 cells. While exposures of 1 µM $AgNO_3$ or AgNP resulted in identical accumulation of Ag in RTgutGC (Fig. 2A), Ag was transported across more efficiently and accumulated more in RTL-W1 cells on exposure to $AgNO_3$ (not shown). A reduction in essential elements (Cu, Zn and Fe) was measured in RTgutGC cells exposed to 3.4 µM of $AgNO_3$. Even though identical amounts of Ag accumulated in RTgutGC cells exposed to 1 µM of $AgNO_3$ or AgNP, cells exposed to $AgNO_3$ induced almost twice as much the metal binding protein, MTb, than cells exposed to cit-AgNPs. In addition, cells exposed to $AgNO_3$ induced Zn and Cu transporters, ZnT1 and ATP7B respectively, while cells exposed to cit-AgNP did not (Fig. 2B). $AgNO_3$ exposure results in activation of MTF1 regulated genes MT and ZnT1 (Günther et al, BBA 2012, 1823) while cit-AgNP appear not to affect this process probably due to different uptake mechanisms, endocytosis *vs* transporter mediated and slower release of ionic Ag. Günther and colleagues show also that Cu and Fe efflux pumps might be regulated by MTF1. This effect could therefore explain the reduction in essential metals in cells exposed to 3.4 µM $AgNO_3$.

Copper ATPase (ATP7A) function was evaluated via monitoring of its trafficking behaviour in human gut cells (Caco2). Exposure to $CuSO_4$ or $AgNO_3$ resulted in redistribution of ATP7A from the Golgi Network to the cells periphery. However, cit-AgNP did not induce such effect (not shown), supporting the hypothesis that at this conditions cit-AgNP do not trigger alterations in Cu homeostasis.

Overall, this study provides new mechanistic understanding on the accumulation and toxicity of Ag and Ti based NPs in RTgutGC cells. The fish intestinal-hepatic cell system has been characterized and proved to be an informative tool to study metal-NP uptake and toxicity. The characterization of the RTgutGC cells as model for fish intestine will be of great value for fish physiology and toxicology studies and it might represent an important tool for assessing the uptake capability and toxicity of different types of compounds. Such tool is in high demand considering the compliance of the European regulation for the registration of new chemicals such as REACH. In depth characterization of cell lines will increase the trust that toxicologist and regulators have of such in vitro systems helping the cause of alternatives to animal testing sustained in the 3Rs (Replacement, Reduction, Refinements).