The main objectives that had to be addressed by the MSCA project HYPERTAM are the following:

(i) Revisit, validate and further expound the tamoxifen - complex III inhibitor effect discovered in our earlier work. (ii) Explore the proposed hypericin PDT - tamoxifen synergistic action in various cell models. (iii) Upon proof of principle of the expected hypericin PDT tamoxifen synergy, evaluate for relevance other photosensitizers with longer wavelength activation like meso-tetrahydroxyphenyl- chlorin (m-THPC). (iv) Investigate the implicated cell death mechanisms in the most striking in vitro results (v) translate the most important PDT in vitro results to their corresponding animal models for a pilot in vivo preclinical assessment.

We have investigated the cell specificity of tamoxifen and the ETC complex III inhibitor myxothiazol. Following a prescreening, the cells that were used as a model in our study (taking also into account the results of prior work) were the MCF7 breast carcinoma cell lines (Estrogen receptor positive, Pasteur metabolic phenotype) and triple negative MDA-MB-231 (estrogen receptor negative, Warburg metabolic phenotype). In specific we focused on the tamoxifen metabolite 4-hydroxy tamoxifen 4-OHT as this was found to be more potent than tamoxifen. We again verified the TAM&MYXO synergy in MCF7 cells whilst there was no synergistic effect in MDA-MB-231 cells. We conducted dose response optimization and we found that the optimal doses were for tamoxifen or 4-OHT 15 μM and for myxothiazol 1.4 μM. We applied the glycolysis inhibitor 2-deoxy glucose (2-DG) on both cell lines studied and we found that although 2-DG increased cell death in MDA-MB-231 cells (Warburg) it did not trigger a synergistic TAM&MYXO effect. MCF7 cells (Pasteur) were not at all affected by the application of 2-DG and the synergy between TAM and MYXO was totally unaffected. So glycolysis, per se, is not involved in the TAM-MYXO synergy.

A general ROS stain (CELLROX green Invitrogen), a dye specific to hydroxyl radicals and a lipid peroxidation imaging dye were applied to the cells and the cells were found to show increased ROS formation in the cases of 4-OHT and 4-OHT&MYXO, increased hydroxyl radicals and increased lipid peroxidation. Unlike the positive control cumin hydroperoxide, which conferred plasmallemal lipid peroxidation, in the case of TAM (&MYXO) the lipid peroxidation was in internal membranes and consistent with mitochondrial localisation.

We have found that apoptosis was not involved in either of the cell lines in the regime followed (15 mM TAM or 4-OHT, 1.4 mM MYXO and assessment at 24h). This was determined by use of flow cytometry with a Terminal deoxynucleotidyl transferase dUTP nick assay. This actually consists a compulsory deviation from our initial plans and there was no meaning of pursuing the apoptosis related research. Using a lactate dehydrogenase leakage assay we found that the method of death was necrosis within 24h. We also found that autophagy was initiated by tamoxifen using western blotting and LC3 antibodies in immunofluorescence confocal microscopy. However autophagy was rather a pro-survival attempt rather than a death mechanism. Fenton reactions which are iron catalysed were found to lead to hydroxyl radicals’ formation. Further with the use of a bc1 Complex III redox assay we showed that 4-OHT and in consequence also its quinoid metabolites can perform reduction of cytochrome c which is accelerated in the presence of complex III. The use of complex I and complex III inhibitors rotenone and antimycin cause abrogation of the 4-OHT MYXO synergy. In low respiration MDA-MB-231 cells increase of the respiration by FCCP uncoupling led to MCF7-like TAM-MYXO synergistic cytotoxicity. **This leads to the conclusion that the MYXO-TAM synergy is mainly due to the semiquinone species formed at the qinonereducing centre of complex III when the quinoloxidizing centre is blocked by myxothiazol.** Other cell lines have been used to verify the results in MCF7 and MDA-MB-231cells and these are MDA-MB-468, T-47D and BT-474 cells. The synergistic effects of MYXO and TAM in MCF7 cells versus additive effects in MDA-MB-231 cells were also verified by isobologram analyses.

We also investigated the mechanism behind HYPERTAM synergism. Hypericin dosing has been established to 2 μM in both cell lines. Light doses have been delivered by a broad-spectrum lamp modified with a hypericin relevant long-pass filter. We also have optimized tamoxifen for this study at 15 μM. MCF7 cells were found to be more resistant to HYP PDT while MDA-MB-231s were more vulnerable. The respective LD50 doses of light were about 30 sec for MDA-MB-231 and 50 sec for MCF7. We have established the synergy of HYP and tamoxifen in MCF7 cells, but not in MDA-MB-231 cells.

We found that the main mode of death was not apoptotic but a combination of autophagy (for which HYP is well documented) and necrosis. Apoptosis was studied by DNA fragmentation in agarose gel. Also in this case the research had to deviate from the pursuit of the apoptotic mechanism, but we found that MCF7 were more resistant to PDT than MDA-MB-231 because of the lack of glutathione peroxidase in the latter, which however possessed glutathione transferase that made them (MDA-MB-231) more drug resistant. We found lipid peroxidation increased in both cell lines following HYP-PDT, TAM or HYPERTAM application. We also coupled metabolic measurements with a seahorse metabolic analyzer to the HYPERTAM strategy and found that although HYP in the dark increased cellular respiration, HYP-PDT and HYPERTAM both significantly decreased the overall cell metabolism (respiration and glucolysis).

Abimodal system (photosensitizer mTHPP linked to a β-cyclodextrin) has been fully tested for their synergy with TAM. In fact the ingenuity of the system is that in this case TAM is incorporated in the cyclodextrin cavity and travels in close proximity with the photosensitizer (mTHPP). It was shown that the efficacy of the system was far above additive effects, and highly synergistic in the case of enhanced-respiration MCF7. This system is already decided to enter animal trials, but outside the project since we wanted to run the all-important HYPERTAM pilot study first. All the above suggest a non-genomic action of tamoxifen especially with increasing cell respiration.

With respect to the *in vivo* studies, both tumour models, MCF7 and MDA-MB-231 have now been established in immunocompromised NOD-SCID-γ mice. HYP PDT and HYPERTAM has been applied to the animals and after several experiments we have identified the optimal HYPERTAM dose. From the preliminary experiments it seems that our optimal dose regime will give us partial response in the MDA-MB-231 mice (we have documented this) and a complete response in the MCF7 mice. These experiments are currently ongoing and will be concluded outside the HYPERTAM project timeframe as they are very interesting for translation.

The expected final results are:

1. A paradigm shift for the clinical use of tamoxifen: instead of a chronic dose, targeted to estrogen positive cancers, a one time (or limited repetitions) high dose of tamoxifen can target high respiration cancers. Since most estrogen positive breast cancers also exhibit high mitochondrial respiration, the clinical use of TAM has to go back to the drawing table as proposed in 2 and 3 below.
2. The non-genomic action of tamoxifen in cells is dependent on the metabolism and more specifically on the respiratory level and redox status of the tumour. This effect can be enhanced by inhibiting the mitochondrial complex III at the quinoloxidising centre and increasing TAM semiquinones production. **Socioeconomic impact: TAM can be used not only for estrogen responsive but for high respiration tumours, in a high bolus dose and not daily small doses, in conjunction with a complex III inhibitor. A new clinical regime, more effective and with less side-effects, may arise where a high bolus TAM is given as an adjuvant to a treatment blocking the respiration in high respiratory capacity cells.**
3. HYPERTAM can largely increase TAM cytocidal effects by blocking complex III in high respiration tumours. **Socioeconomic impact: Although hypericin is perhaps the most potent photosensitizer, HYP-PDT has not been used in the clinic so far due to the limited tissue penetration depth of the light, at the HYP excitation wavelength (~600 nm). With the assistance of a high tamoxifen bolus as adjuvant, cancer cells that are not killed but their respiration blocked by HYP PDT, will be finished off by TAM.**