FINAL REPORT

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Summary of the EDOCAL Project

Globally there are 24.6 million people living with cancer. Cancer is a leading cause of death worldwide. Deaths from cancer in the world are projected to continue rising, with an estimated 9 million people dying from cancer in 2015 and 11.4 million dying in 2030. In Europe there are 3 million new cancer cases per year (38 countries), with 2 million new cases in the EU25 alone. This represents 340 new cases / 100000 people per year. One in three men and one in four women will be inflicted with cancer during their life. There is no doubt about the fact that cancer is an increasingly important factor in the global burden of disease with tremendous economic impact.

The U.S. National Institute of Health (NIH) estimated the overall annual costs for cancer in 2000 at €112 billion in the U.S. alone, with €38 billion for direct medical costs and the rest as a result of lost productivity. These figures can, in their opinion be doubled to cover the rest of the developed world. The total global cost of cancer in 2000 was €150 billion. The figure for direct medical costs alone per patient in Europe is €31.000 and rising .

According to the WHO the burden of cancer can be reduced by systematic and equitable implementation of evidence based strategies for cancer prevention, early detection and management of patients with cancer. Up to 33% of the cancer burden could be reduced by implementing preventative strategies aimed at reducing the exposure to cancer risks. Another 33% of the cancer burden could be reduced if detected early and treated adequately. The WHO concludes that, early diagnosis of cancers could save the developed world an average of €25 billion per year on medical costs and $50 billion per year on realized productivity. The direct savings on medical treatment alone would be more than €10.000 per patient if early detection was possible.

The purpose of the EDOCAL project is to create a breakthrough tool for early cancer detection by combining state of the art laser and imaging technology with leading medical research (expertise and hands on practical medical treatment experience). We expect that the results from the medical research will enable the SMEs to create a device that can be used for first line cancer detection, making early cancer detection more accessible to more people all over the world. This will create a new mass market for the products created by the SMEs and will ensure for them a position in a new and quickly growing market: Cheap, reliable, medical diagnostics for everyone.

In spite of the huge increase in certain diseases attributed to increased prosperity, growth in product sales (e.g. endoscopes) for treatment of these diseases has not happened at the same rate. Reports by Frost & Sullivan indicate the main reasons for the slow growth to be: lack of new and technically exciting products, the high cost associated with initial purchase of endoscopes and resistance to new technologies. EDOCAL aims to provide the innovative breakthrough, at reasonable costs by combining advanced, proven and low cost telecom and state of the art semiconductor technologies with the latest advances and insights into medical procedures provided by the RTD partners. EDOCAL also aims to enable the European companies to differentiate themselves from the high-end segment players in Japan and the US through the use of innovative features and will increase the technology lead of Europe compared to the low cost OEM players from China and India.

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1 EDOCAL Publishable Summary

Early detection of cancer saves lives, improves quality of life and reduces health care cost.

In Europe there are 3 million new cancer cases every year. According to the WHO the burden of cancer can be reduced by systematic and equitable implementation of evidence based strategies for cancer prevention, early detection and management of patients with cancer. 33% of the cancer burden could be reduced if detected early and treated adequately. The WHO concludes that early diagnosis of cancers could save the developed world an average of €25 billion per year on medical costs and €50 billion per year on lost productivity.

Early cancer diagnosis today consists of recognition of some of the early signs by the patient themselves or detection from screenings carried out on specific population groups. If something suspicious is found, the next step is to perform a blood analysis and/or to look with non-invasive imaging techniques. Current imaging techniques are widely used in diagnostic procedures but they cannot give conclusive evidence of cancer. Today, only a biopsy and the subsequent pathologist's interpretation can give a definitive diagnosis of cancer and this is not always sufficient. In the EDOCAL project the Proof of Concept has been achieved for early detection of cancer based on blue laser technology. The technology has been tested and proven for oesophageal cancer detection and has the potential for early detection of many other types of cancer including stomach and colorectal. The technical principle behind the EDOCAL concept is as follows.

In cancer, tissue cells tend to create additional blood vessels to support their growth. The molecule protoporphyrin (PpIX) is generally present in blood vessels and exhibits red fluorescence when excited with blue light in the 375-425 nm range. Cancer cells are detected by observing the red fluorescence at a matching excitation wavelength. The wavelength of the laser has to be tuneable as the matching wavelength depends on the person, type of illness and presence of other chemicals. To increase the intensity of the red fluorescence, a porphyrin prodrug can be administered either orally or locally which synthesizes PpIX in the cancerous (cells) tissue. By using a tuneable laser system and combination of endogenous fluorescence (as a result of extra blood) and exogenous fluorescence (produced by administration of a porphyrin prodrug), it is possible to accurately distinguish between normal and cancerous tissue.

The results of the EDOCAL project can be summarized as follows:

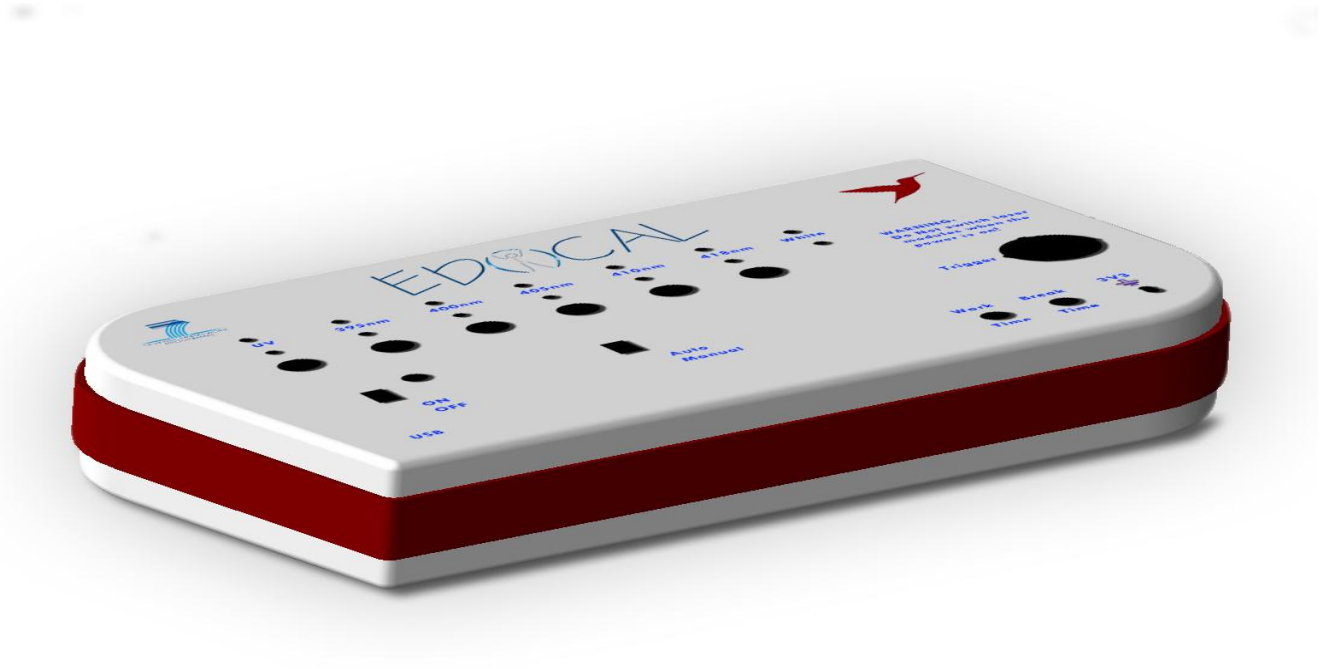
- A tuneable blue laser prototype was defined and the system built for research purposes.
- Experiments to detect cancer using shifts in excitation and emission wavelengths were carried out in 70 patients and we obtained a total of 1557 spectra from 290 sites in the oesophagus. Using the prototype, each site was illuminated with multiple wavelengths followed by registration of the corresponding auto-fluorescence spectrum and tissue biopsy for histological correlation.
- Results from the EDOCAL prototype show that it is possible to discriminate early cancer with 80% sensitivity and 81% specificity, which meets the performance requirements targeted at the start of the EDOCAL project.

- Proof of concept has been achieved for early detection of cancer based on a tuneable blue laser technology.
- Preliminary specifications for the first demonstrator have been defined.
- The first Patent application has been filed and new filings for the latest results are being worked on.
- We have demonstrated that the lasers used can be mass produced.

The EDOCAL project combined state of the art laser and imaging technology with leading medical research, expertise and hands on practical medical treatment experience to validate the first prototype and investigate whether or not it not it would be effective in discriminating early cancerous tissue from healthy tissue. The project has succeeded in establishing proof of concept for oesophageal cancer detection. The technique was validated using cell lines in vitro before applying the procedure to patients.

2 EDOCAL Project LOGO

3 EDOCAL Product Design



4 Summary of Results from WP2

Objectives

To ensure that all project results are formulated and compiled into a protectable form and that all necessary patents are filed. To transfer specific knowledge from the RTD performers to the SME participants to enable them to apply and embed the research results into the laser systems in such a way that the best possible product devices result. To broadcast the benefits of the developed technology and knowledge beyond the consortium to potential industrial users and the extended medical community worldwide. To assess the socio-economic impact of the generated knowledge and technology.

Conclusions

In the past years many principles of tuning for tuneable lasers have been patented because of the predicted need for telecom applications. In the visible wavelength range many patents have been filed using the external grating concept. The conclusion was that it is not to be expected that patents can be generated in the field of tunable lasers. This conclusion is also supported by discussions with multiple patent attorneys. Patenting possibilities could only be expected in combination with the application.

The conclusion from the searches that were done on the application area were that no patent and no publication exactly described the use of the excitation peak shift for tumour diagnosis which is why we felt a patent application, based on the results of the project, was warranted. The first patent was filed on 30.12.2011. The second patent was filed on 23.02.2012.

Knowledge dissemination

This is on-going.

Three manuscripts are being prepared at the moment and it is expected that they will be offered for publication later this year. It was agreed that the patents should first be filed before any publications were done.

Social Impact

Early cancer diagnosis today consists of recognition of some of the 'early' signs by the patient themselves or detection from **screenings** carried out on specific population groups. Typical signs are: lumps, sores, persistent indigestion, persistent coughing and bleeding from the body's orifices. If something suspicious is found the next step is to perform a blood analysis and/or to look with non-invasive imaging techniques. Current imaging techniques are widely used in diagnostic procedures but they cannot give conclusive evidence of cancer. **Today, only a biopsy and the subsequent pathologist's interpretation can give a definitive diagnosis of cancer** and even sometimes this is also insufficient. A biopsy is a procedure to remove a piece of tissue or a sample of cells from the body so that it can be

analysed in a laboratory. There are five different types of biopsy procedures depending on the type of cancer suspected and the location of the suspicious cells.

The main problems with current cancer diagnostic techniques and procedures are:

1. **The lack of early primary detection methods.** Early detection depends on the ability to discriminate healthy from (potentially) cancerous tissue, before physical symptoms like sores and ulcers start to appear. In the early stages when the impact of many cancer's is still minimal, it is difficult and often impossible to conclusively discriminate healthy from malignant tissue, leading to incorrect/inconclusive results.
2. **High costs due to late interventions**. When cancer is not detected in an early stage, complicated surgical interventions, chemotherapy and radiotherapy with limited long term success rates become necessary. The direct and indirect costs are huge when compared to the costs associated with an early diagnosis and the resulting intervention which are sufficient if the cancer has not had a chance to mature and spread. For example, early diagnosis and treatment of gastro intestinal (e.g. Barrett's) cancer has a high success rate via endoscopic intervention. However **when detected late**, surgery is always necessary and chances of success are low. At this stage there is **a mortality rate of 3-5% during surgery and a survival rate of only 20% after 5 years**. As stated earlier, direct savings per patient per year would be approximately €10.000. In the EU alone 1.48 million new cancer cases are handled, every year, of which 25,000 are cancer of the Oesophagus. This adds up to a saving in direct cost of about €250 million per year due to intervention at an earlier stage.
3. **Sensitivity and specificity of existing imaging techniques are too low**. MRI and CT scans when used for the types of cancer that are most difficult to detect, have a sensitivity and a specificity of roughly 40-70% and early neoplastic lesions are often too small to be detected by these scans. Sensitivity and specificity of existing endoscopic procedures is roughly 50-80%. This means that there is insufficient contrast between the healthy and cancerous tissue, making it impossible to make a reliable diagnosis. Therefore a biopsy with all of its drawbacks is still the golden standard for conclusive cancer diagnosis.
4. **Non-existence of reliable red flag tools.** Typically the sensitivity of existing tools is between 60-70%, which is not reliable enough to be of much use in daily medical practice. Today no online surgical tools exist that can clearly and immediately discriminate healthy from cancerous tissue over a large surface area. Oncologists agree that a wide angled, easy to use, real-time detection tool small enough to be integrated into an endoscope would be an enormous help to them, showing them where to intervene and allowing them to track their progress. Such a tool does not exist today but the results of this project bring us a step closer to being able to create one.
5. **The conclusive biopsy lab analysis is slow.** Typically is takes about 1 week before results of the biopsy are available and another 1-2 weeks before the next treatment step can be taken or appointments scheduled. Furthermore there is the risk that the wrong tissue is sampled for biopsy (sampling error) and that too little malignant tissue or too much healthy tissue is removed from a patient.

The main cause of Barrett's oesophagus is thought to be an adaptation to chronic acid exposure from reflux oesophagitis. In the last 40 years, the incidence of oesophageal adenocarcinoma has been increasing in the Western world. Barrett's oesophagus is found in 5–15% of patients who seek medical care for heartburn (gastroesophageal reflux disease, GERD), although a large subgroup of patients with Barrett's oesophagus do not have symptoms. It is considered to be a premalignant condition because it is associated with an increased risk of oesophageal cancer (more specifically, adenocarcinoma) of about 0.5% per patient-year.

Diagnosis of Barrett's oesophagus requires endoscopy (more specifically, oesophago-gastroduodenoscopy, a procedure in which a small camera is inserted through the mouth to examine the oesophagus, stomach, and duodenum) and biopsy. The cells of Barrett's oesophagus, after biopsy, are classified into four general categories: non-dysplastic, low-grade dysplasia, high-grade dysplasia, and frank carcinoma. High-grade dysplasia and frank carcinoma patients are generally advised to undergo surgical treatment. Non-dysplastic and low-grade patients are generally advised to undergo annual observation with endoscopy. In high-grade dysplasia, the risk of developing cancer might be at 10% per patient-year or greater. In specific cases endoscopic evaluation of patients is recommended to detect early neoplasia at a curable stage. We have shown that the combination of refined endoscopic ultrasonography and laser fluorescence techniques can lead to earlier detection, more precise localization, and even curative ablation of gastrointestinal malignancy. In addition, the EDOCAL technology could render the biopsies obsolete as diagnosis could be done using the tool rather than having to cut out tissue and send it to pathology.

In the short term the most immediate benefit to the cancer patients is during cancer surgery. Surgery is in 80% of all cancer types still the most used treatment procedure. EDOCAL can thus benefit more than 2.4 million people per year in the EU alone. The PCD can be beneficial for all surgery treatments as it will enable the specialist to instantly characterize the cancer type and maturity, while in real time progression of cancer surgery can be tracked. Today when there is doubt about the extent of the cancer cells surgeons remove more tissue than necessary just to be sure. Once a device becomes available, as a result of EDOCAL, that can in real-time inform the surgeon as to whether tissue is malignant or not, the amount of tissue being surgically removed can be minimised, leading to shorter recovery time for patients. This is not possible with existing technologies. EDOCAL will in addition to earlier cancer detection boost the success rate of cancer surgery.

In the case of colorectal and stomach cancer evidence exists showing that auto-fluorescence spectroscopy is a promising technique for the endoscopic in-vivo diagnosis of these cancers. The sensitivity and specificity of existing tools is currently between 60-70%, which is not reliable enough to be of much use in daily medical practice. Today, no online surgical tools exist that can clearly and immediately discriminate healthy from cancerous tissue over a large surface area. Oncologists agree that a wide angled, easy to use, real-time detection tool small enough to be integrated into an endoscope would be an enormous help to them, showing them where to intervene and allowing them to track their progress. Such a commercial tool does not exist today but the results from the EDOCAL project have brought us a step closer to creating a new class of medical devices to address this issue.

The proof of principle has been realised for oesophageal cancer and we plan at a later stage to apply the same concept to other cancer types starting with stomach and colorectal.

The most important result of this project is the tremendous improvements that can be made to the quality and speed of care provided to patients. By detecting earlier, earlier intervention is made possible. This will lead to more successful medical procedures, reduction of complications and suffering for patients and significantly lower costs. In Europe alone each year 1.5 million new cancer cases are detected and everyday 3000 people die of cancer, making it the 2nd cause of death in Europe. The results of this project will provide a breakthrough in early detection resulting in savings of up to €10.000 per incidence per year. For the new cancer cases this will be a saving of at least €15 billion in direct medical costs each year. The impact on indirect costs, due to loss of workforce is orders of magnitude larger.

The second benefit is that it can be used to provide the medical community with a practical tool that can easily and conveniently be used in day to day medical practice, allowing them to do their work quicker as no biopsy is needed and improving the quality and accuracy of their work as they now get the tools needed to instantly discriminate cancerous from healthy tissue.

5 Summary of results from WP3

WP 3 Key results

The purpose of this task was to define and build a tuneable fluorescence spectroscopy system. The excitation wavelengths should be tuneable within a wavelength range of 365-450 nm. Light was directed to the sample via a bifurcated optical fibre. The Fluorescence emission was collected by the same fibre and spectrally resolved using a diode array spectrometer. The emitted signal was analysed using a laptop computer with commercial software. The system was tested using solutions of a photo-sensitizer, and also using photo-sensitizer-treated cell lines. The specification for the first system was defined by the RTDs and this first system (called the Dynamic Blue system) was built by the SMEs and delivered to the RTDs for testing.

A tuneable optical fluorescence spectroscopy system was constructed. Light was directed to the test site via a bifurcated optical fiber. Fluorescent emission was collected by the same fiber and spectrally resolved using a diode array spectrometer. Stray light is eliminated by use of optical filtering. The emitted signal is analyzed using a laptop computer with commercial software. The Dynamic Blue device NW1 has been shown to be electrically safe. The optical radiation emissions have been accurately characterised.

A system has been demonstrated that is capable of reproducibly detecting fluorescent signal derived from pro-drug induced proto-porphyrin in human cultured cells. In addition, a quantitative difference in fluorescence intensity was observed with time after exposure to the prodrug, consistent with that found using other instruments and also reported in the literature.

The specification for the system to be used at AMC was identical to that for NWH, however the use was different. The AMC focussed on ex-vivo measurements on endoscopic resection specimens for the characterisation of specific tissue (auto)fluorescence. Therefore, a set-up had to be developed to investigate freshly obtained EMR (endoscopic mucosal resection) specimens. To closely resemble an in-vivo setting, a fibre optic probe was specially designed to deliver the excitation signal to the tissue through the working channel of the endoscope and to carry back the fluorescence signal to the connected spectrometer and computer for spectral analysis. The endoscope, probe and spectrometer were fixed on a bench and the EMR specimen was placed in an oblique angle below the tip of the probe, thus mimicking the in-vivo endoscopic setting.

Conclusions

The test set-up was tested successfully. The system demonstrated that it was capable of reproducibly delivering and detecting a fluorescent signal derived from either proto-porphyrin IX solutions or pro-drug induced cell lines. The findings were consistent with those found using other instruments and also reported in the literature.

6 Summary of Results from WP4

Fluorescence in Model Biological Systems and Cell Lines

WP 4 Key results

The aim of WP4 was to study factors that influence differences in fluorescence intensity and determine the optimum excitation conditions to allow differentiation between normal and cancerous cell lines.

The photosensitiser environment influenced the absorption peak maximum and shape for both PpIX and Foscan. In terms of fluorescence, 1:75 liposomal solutions of PpIX most closely reflected the data obtained from ALA-treated normal human skin. The fluorescence intensity of photosensitisers presented to NW2 – the second Dynamic Blue iteration - device in different environments was in agreement with data previously published using traditional spectrophotomeric means. The power adjusted measurements of PpIX concentration taken with NW2 over the range 0.1-1.0 μM (consistent with concentrations found in human skin following application of ALA) were linear and in line with measurements made using the Hitachi F2500 spectrophotometer. The limits of 'quantifiable' detection (S:N > 10) of PpIX and Foscan fluorescence by NW2 were comparable to the Hitachi F2500 (< 0.1 μM). NW2 was actually more effective at detecting Foscan fluorescence in PBS compared to the Hitachi F2500 (fluorescence not detected). The precision of measurements of photosensitisers presented to NW2 in different environments was good as indicated by the %CV. The %CV of the fluorescence maxima was generally in the region of 0.03%. Peak intensity measurement was least precise in solutions that encouraged aggregation of the photosensitisers, and most repeatable in liposome suspension at less than 5%. In the biologically mimicking solutions of BSA, FCS and liposomes, tuning the excitation wavelength of PpIX from 400-410 nm shifted the emission peak maximum by 0.2, 0.5 and 0.3 nm respectively. For the chlorin these shifts were smaller (0.2, 0.2, and 0.1 nm respectively). Thus the porphyrin seemed to be more affected by tuning the excitation wavelength than Foscan (Refer to D4.1 and 4.M4). In human skin, fluorescence from endogenous chromophores (autofluorescence; AF) shifts by 8.7 nm from 513.9 nm to 522.6 nm when the excitation wavelength was changed from 400 to 410 nm.

Cell-specific differences in fluorescence were observed between cell lines derived from the epidermis (HaCaT), oesophagus (OE19), brain (SHSY5Y) and bladder (HT1197). All cell types were able to effectively convert ALA or MAL to PpIX over time with the more transformed the cell phenotype accumulating more cell-associated PpIX. More porphyrins consistently accumulated in OE19 or HT1197 cells than in HaCaT or SHSY5Y cells. The majority of porphyrin was PpIX, except in OE19 media, where coproporphyrin (4-COOH) was the most dominant after 24 hours. Spectroscopic analysis (I620/I635) using the NW3 – the third Dynamic Blue iteration – device illustrated a prominent 620 nm peak in OE19 cell media, which could be 4-COOH. This 620 nm peak was associated with some (OE19, HT1197) but not all (SHSY5Y, HaCaT) tissue cell types. Factors that influence the differences observed between these cell lines were investigated, in particular, PpIX efflux and haem synthesis. The present results do not suggest a major impact of the porphyrin transporter protein within the clinically relevant incubation time used in this study. Distinct differences in fluorescence emission at 620 and 635 nm may thus

indicate progression towards a more cancerous cell type. All excitation wavelengths were able to induce adequate fluorescence signals, except the 367 nm LED. PpIX fluorescence peak intensity shifts were observed between 4 and 24 hours, with all cell lines; the highest in OE19 and HT1197 cells. These cell lines also accumulated the highest cell associated concentrations of PpIX. Emission wavelength shifts, however, where not detected between excitation wavelengths or between cell lines. Finally, autofluorescence from cells could not be observed.

Blue visible light up to a dose of 5 J/cm^2 was not in itself phototoxic or photogenotoxic to cell types on its own when compared to baseline (dark) values. Exposure of ALA treated cells to light resulted in a small but significant increase in DNA strand breaks, however no effects on cell survival were subsequently observed 24 hours later in the phototoxicity assay. The doses that were used encompassed and exceeded those that might be delivered to tissue by NW3 in its current form, and changes started to be seen in the gel electrophoresis assay at doses of 80 mJ/cm^2. Non-light exposed cells did not exhibit any toxicity. The data confirm the 'desired' specification for a device emitting no more than 1 mW/cm^2 or higher. It is also notable that no measurable changes were seen in the phototoxicity assay, (which is particularly vulnerable to damage to the cell membranes) or in the morphology of the cells.

NWH successfully determined fluorescence in model biological systems and cell lines, and completed all the tasks set without major revision to the agreed description of work, with NWH contributing significantly to the EDOCAL project.

These results have been presented at two scientific conferences (British Medical Laser Association (BMLA) Annual Conference, Woburn, UK and European Society of Photobiology (ESP), Geneva, Switzerland), and an article for peer-reviewed publication entitled "5-aminolaevulinic acid- and methyl-aminolaevulinic acid-induced porphyrin synthesis and intracellular localization in cancer cell lines" is currently in preparation.

7 Summary of Results from WP5

The content of this work package was to use the test setup for the ex- and in-vivo spectroscopy in Barrett's patients with and without dysplasia as well as the 5-ALA induced PpIX fluorescence in human adenocarcinoma OE19 cells and human tissue with and without dysplasia.

D3: Specification and test systems

The Dynamic Blue prototype was developed by the SME partners and evaluated by the AMC, regarding the appropriate specifications for the objective of the medical research as well as the application in a basic scientific research or (pre-)clinical setting.

The AMC developed an ex-vivo and in-vivo test set-up for the Dynamic Blue prototype, comprising a specially designed optical fiber probe, custom made driver software and a set-up for the application of the prototype during real-time endoscopic procedures.

D5.1: Analysis of biopsy and EMR specimens from patients with BE

Fluorescence and reflectance spectra generated by the Dynamic Blue prototype at several wavelengths were obtained from patients with Barrett's esophagus with and without dysplasia. In a first step tissue from endoscopic mucosal resection was used to validate the Dynamic Blue prototype in an ex-vivo setting. After validation the prototype was applied to more than 70 patients during standard endoscopy at the AMC Amsterdam.

Collected fluorescence spectra were analyzed using two distinct methods: emission intensity analysis and spectral shape analysis. Fluorescence spectra obtained using a single excitation wavelength and combinations of spectra obtained using different excitation wavelengths were analyzed to find the optimal (combination of) wavelength(s).
All autofluorescence spectra were corrected by the background signal and normalized with respect to the total intensity of the spectrum.

Ex-vivo: 11 endoscopic mucosal resections (EMR) were performed in seven patients with dysplasia in Barrett's oesophagus BO. All patients underwent an EMR using the EMR-cap technique of both a neoplastic and a non-neoplastic area. Immediately after resection, the EMR-specimen was pinned on a paraffin block for direct ex-vivo examination. The Dynamic Blue prototype was activated (405 nm) and ex-vivo autofluorescence spectroscopy and corresponding histology sampling were subsequently performed on a total of 26 mucosal areas in Barrett's mucosa in these 11 EMR specimens. The ex-vivo measured autofluorescence spectra of normal Barrett's and HGIN/EC showed differences in the relative intensity and in the spectral shape.

In-vivo: in-vivo autofluorescence spectroscopy was performed in 2 phases. In phase 1 and 2, a total of 70 patients was included, in whom a total of 290 areas was investigated with in-vivo autofluorescence spectroscopy using one of the Dynamic Blue prototypes. Comparing the results from intensity and shape analysis, the intensity ratios show a higher significance in discriminating non-dysplastic Barrett (IM) from

grouped high-grade intraepithelial neoplasia (HGIN) and early adenocarcinoma (CA). Several intensity ratios of the emission spectra were tested for each excitation wavelength and double intensity ratios were calculated for all possible combinations of excitation wavelengths.
The results in phase 1 showed a sensitivity of 100% and a specificity of 67% for single excitation with 365nm using white light corrected autofluorescence spectra. The combination of 365nm and 405nm excitation improved the sensitivity from 67% up to 100% (IM: 1, HGIN/CA: 3). The number of used spectra in phase 1 was limited due to the early stage of prototype development.
Based on these results, the optimal algorithm is composed of white light corrected autofluorescence spectra, obtained with 365nm and 405nm excitation, for calculating a 'double intensity ratio' at 560nm and 640 nm.

In phase 2 the developed algorithm of phase 1 was validated on a new set of data which was obtained using an improved prototype. Sensitivity and specificity for single excitation was 75% and 73% respectively.
For white light corrected autofluorescence spectra analysis on the new data set the combination of 365nm and 405nm did improve the sensitivity and specificity compared to single excitation. A sensitivity of 80% and a specificity of 81% was reached by calculating the double intensity ratios at 495nm and 560nm. The student t-test showed a significant difference between non-dysplastic Barrett (IM) and grouped high-grade intraepithelial neoplasia (HGIN) and early adenocarcinoma (CA).

D5.2: Analysis of biopsy and EMR specimens from patients with BE
In order to execute the work tasks of D5.2, a standardized in-vivo model was developed, using the chorioallantoic membrane (CAM) model, The CAM model is a well established model in the literature and the AMC Department of Biomedical Engineering and Physics has extensive experience in performing CAM experiments. However, transplanting whole tissue biopsies for the purpose of this study requires adaptations of known procedures and the development of new methods. The CAM model was adjusted and optimized for the EDOCAL project.
20 eggs were used to investigate optimal egg handling and growth conditions. A total of 135 eggs were used to develop the AMC CAM model: 79 biopsies out of 22 patients (18 males, mean age 67 years) and 24 cell samples were transplanted on 109 eggs.
Our results show that the developed AMC-CAM model is a feasible model for the study of 5-ALA induced PpIX fluorescence. As planned, patient measurements were subsequently conducted with this CAM model, as well as fluorescence studies on cell lines. For all measurements the developed set-up for patient measurements with the Dynamic Blue prototype and the designed optical fibre were used.

Human adenocarcinoma (OE19) cells were obtained from Ninewells Hospital Dundee (NWH) and cultured according to the NWH protocol, followed by transplantation on the AMC CAM model. PpIX fluorescence intensities (I_{636}/I_{600}) were measured at 4.5, 6 and 28 hours after topical application of 5-ALA. An increasing trend for PpIX fluorescence intensity (ratio I_{636}/I_{600}) with increasing excitation wavelength and changes over time was observed. Highest PpIX fluorescence intensities were obtained either with 410nm or 416nm excitation at all time points. At 6h, PpIX fluorescence showed the highest

intensities, whereas at 28h after 5-ALA administration lowest PpIX intensities were observed. These results were not statistically significant.

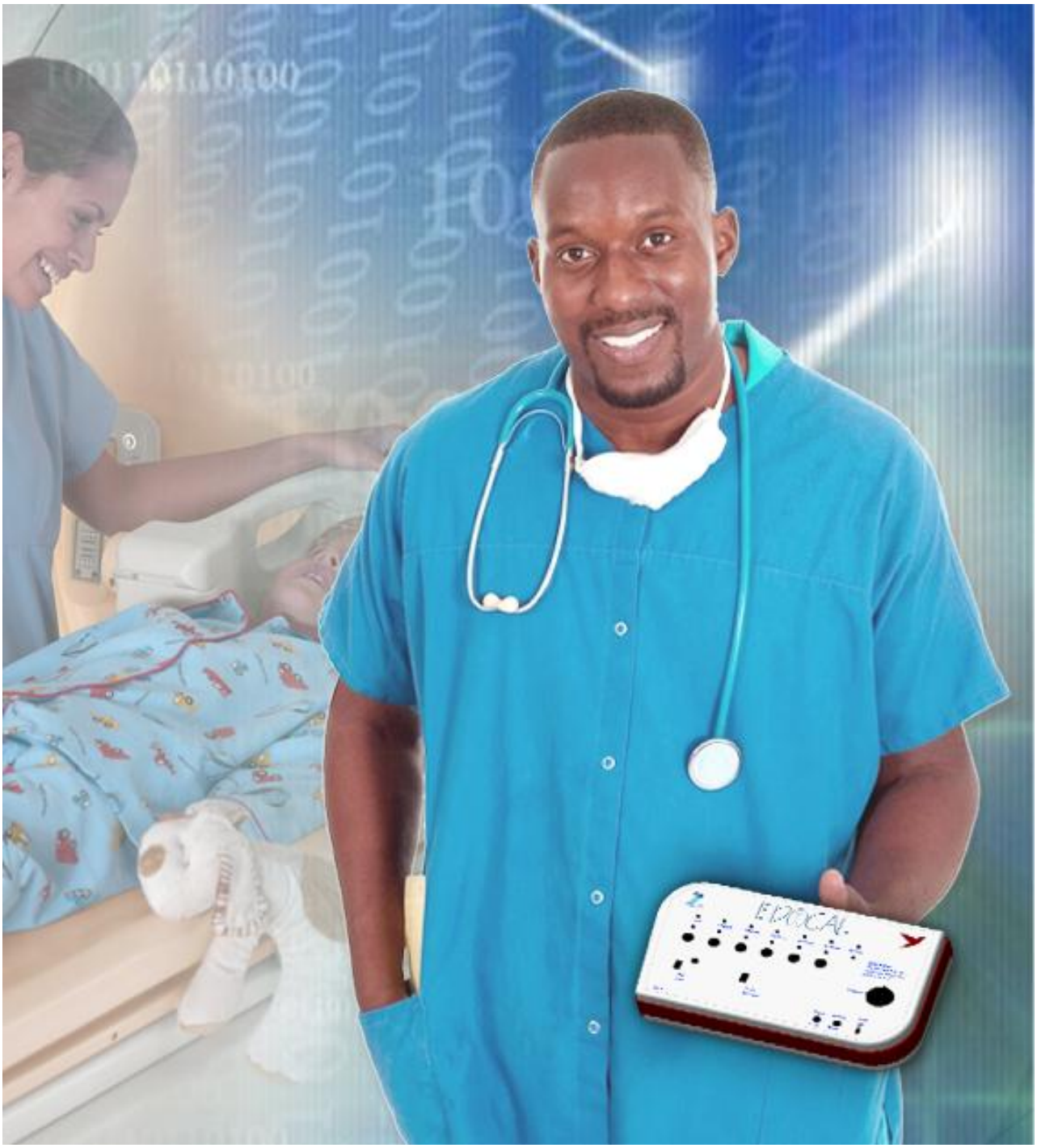
Biopsy specimens were obtained from patients with non-dysplastic and dysplastic Barrett's tissue and immediately transplanted on the CAM. The PpIX fluorescence intensity (I_{636}/I_{600}) after 5-ALA administration for non-dysplastic and dysplastic Barrett's tissue was studied between patients (inter-patient) and for individual patients (intra-patient). The results for all obtained data (inter-patient) on all averaged PpIX fluorescence intensity ratios (I_{636}/I_{600}) per tissue type, showed no significant difference between non-dysplastic and dysplastic Barrett's tissue. The excitation wavelengths 410nm and 416nm showed a trend towards highest PpIX fluorescence in all tissue types. The 410nm excitation looked to have the highest difference between non-dysplastic and dysplastic PpIX fluorescence at all time points. Furthermore dysplastic tissue tended to have higher intensity ratios than non-dysplastic at all time points and all excitation wavelengths. These trends were not statistically significant.

Per patient (intra-patient) analysis of the 5-ALA induced PpIX fluorescence intensities showed higher fluorescence intensities for dysplastic tissue compared to non-dysplastic Barrett's tissue. The longer wavelengths 410nm and 416nm give the highest intensity ratios for both types of tissue.
In 45% of the examined cases a shift in excitation peak was observed. The peak shift occurred between 410 and 416nm from non dysplastic to dysplastic Barrett's tissue. However, the shift did not show a specific direction. PpIX fluorescence of both the cells and tissue was always observed at 636nm.

Beside the prominent PpIX fluorescence peak at 636nm, another peak at 620nm was observed.
Additional analysis of this peak was performed by calculating the ratio I_{620}/I_{636} for the spectra obtained from cells and both tissue types. The results show an increase of 620nm fluorescence intensity over time which is consistent in cells and human tissue. Furthermore the intensity increases at shorter excitation wavelength. In addition, the presence of the 620 nm was also linked to the appearance of a 680 nm peak.

In summary, the PpIX fluorescence analysis for all obtained data (inter-patient) showed no significant difference between non-dysplastic and dysplastic Barrett's tissue. The intra-patient analysis of the PpIX intensities showed higher fluorescence intensities for dysplastic tissue compared to non-dysplastic Barrett's tissue. In 45% of cases, an excitation peak shift was observed. However, the direction of the shift was found to be independent from the tissue type. The longer wavelengths 410nm and 416nm were most efficient to distinguish between non-dysplastic and dysplastic Barrett's tissue. In addition another emission peak at 620nm was observed and could potentially be used to further improve the discrimination of different tissue types.

8 Summary of Results from WP6



Summary of key results

Creating a tunable blue laser is possible at this moment. Creating a tunable laser that operates over a range of 25nm is not feasible in the short term. However, this was not an obstacle for the project as we have seen from the results of the research. The same results can be achieved using a combination of tunable lasers instead of just one.

Combining Vernier tuning in a number of conveniently spaced laser diodes at different wavelength provides the most practical solution for achieving continuous tuning over a large wavelength range, in the short term.

Specification of the wavelength range required

Originally the required wavelength range was defined as 405nm to 430nm. During the first phase of the project we widened the range to cover 365nm to 445nm at the request of the RTDs. After the first tests were carried out, it was agreed to narrow this range again with the lower limit being 390nm and the upper limit being 425nm. After the results of the research by AMC and NWH in the third quarter of the project, it was agreed to choose 405nm as the central wavelength in the tuning range, meaning that the new range is defined as 395nm to 415nm. Most recent results indicate that the upper limit should be about 420nm.

Conclusion: Due to the limits of the current technology for blue lasers we have had to change the architecture of the device from one tunable laser operating over 25 nm to 5 tunable lasers, each operating over 5 nm. **The final range agreed upon is 395nm to 420nm**

The final prototype system

In order to supply the light via an endoscope or other device to the tissue, the light from the lasers had to be combined into one delivery fiber. The prototypes that were delivered to the RTDs already incorporated this coupling system.

The light of an individual laser is coupled into a 100 micron core fiber and the 5 fibers of the lasers are attached to a 400 micron core fiber which can be connected to an endoscope delivery fiber. The required light output from each individual laser should be around 10 mW coming out of the delivery fiber.

The total time sequence for wavelength switching and tuning should be limited to 1 second for the complete wavelength range.

The laser system is equipped with a 400 micron core fibre with an SMA connector. The activation of the lasers for switching and tuning can be done in a fixed sequence or manually.

Spectral emission profiles from each diode have been accurately characterised. Adequate output powers were recorded. The radiant powers were noted to be sufficient for induction of fluorescence in

photo-sensitizer solutions and cell lines, except in the case of the 367 nm LED. The wavelength response for each optical instrument available at NWH was assessed using a mercury lamp, which is routinely used for wavelength calibration purposes. The mercury lamp lines were consistently reproduced by each instrument and when compared to each other, a COV of less than 1 % was achieved. Alterations to the optical delivery and detection system were undertaken. These involved modifying the optical fibre configuration. This was important as optical fibres by themselves have the potential to produce interfering background fluorescence signals. The NW3 – the third dynamic blue iteration - device demonstrates sufficient reproducibility and usability. Although the NW3 device requires further optimisation, it produced acceptable fluorescence signals that were necessary to complete the project.

The requirements for the control application and software user-interface were considered based on input from the medical experts, the end-users. It is important to ensure that system requirements for acceptance in the field are included in the software platform.

The software developed at the AMC has progressed during the project towards a flexible and user-friendly application, also including data safety and reporting considerations. We have distinguished between information that is needed as a real-time feedback to the user, such as quality of the spectrum and status of the lasers and information that can be processed after initial diagnostic measurements in the patient.

A complete User Requirements Specification (URS) for the final product has been completed.

9 Summary of Results from WP7

Plan for the use and dissemination of foreground

AMC and Ninewells both are participating in leading global networks in their fields. They will help to endorse and indirectly promote use of the EDOCAL system during congresses, seminars and in publications. Both RTDs are seen as leading in their field which will make the task of introducing the results wasier.

Due to the fact that two patents were developed during the project, the first one was filed on 30.12.2011 andthe second one on 23.02.2012, the RTDs have agreed to postpone their presentations at (international) conferences. Presentations at the first international conferences are scheduled for 2012. Three manuscripts are in the process of being completed at this moment and they will be published during 2012.

During these seminars and congresses detailed information on paper will be available including product leaflets, underpinned with medical publications and studies from AMC, NWH and other leading medical specialists. This information will also be made available via the dedicated website (www.edocal.eu).

Wide public awareness: patient organizations, press and nurse organizations

Another important stakeholder is a much wider audience of patients, patient organizations, press and nursing organizations. In the future we will make information about the benefits of this new technology available through various means. The website will continue to be updated as new information becomes available.

Once we have built and tested the first demonstrators, commercial presentations endorsed by leading medical experts will be held at leading congresses where patient organizations and the press come together. Examples of such congresses are Medica in Dusseldorf. The current systems are not yet good enough to do this. 2M and AMC are working on optimisting the algorithms and CST and TopGaN are working on industrialising the lasers. NWH and AMC are further investigating the new emission peaks that were found by both of them independently. More publications on this will follow.

In summary, the potential impact of the results of the EDOCAL project can be huge in terms of medical diagnostics, improved quality of life of patients and carers and direct and indirect economic savings. In order to realise the potential impact the SMEs are committed to taking the next steps: to build demonstrators which can be given to medical centres for testing and validation and to developing the necessary partnerships in order to commercialise the results and make them available on a large scale.

10 Common ground results from NWH & AMC

Throughout the EDOCAL project, the RTD performers, NWH and AMC, have aimed to determine fluorescence in model biological systems, cell lines, biopsy and endoscopic resection samples.

Together NWH and AMC have shown that an adequate fluorescence signal was produced from all light sources available from each Dynamic Blue prototype. All light sources – with excitation wavelengths ranging between 369 – 416 nm - were successful in generating ALA-induced PpIX fluorescence signals. PpIX fluorescence was assessed by employing multiple excitation wavelengths; however, irrespective of cell or tissue type, a shift in fluorescence emission wavelength of the main emission peak was not observed and remained anchored at approximately 636 nm. This highlights a difference between protoporphyrin produced endogenously from endogenous ALA, preformed protoporphyrin added exogenously, and protoporphyrin produced exogenously from exogenous ALA. This may warrant further investigation. PpIX fluorescence intensity was time-dependent (i.e., 0-28 hours), and these findings were evident regardless of OE19 cell growth conditions, for example, as either an *in vitro* monolayer or after transplantation to a chorioallantoic membrane (CAM) model.

Both RTD partners observed a notable peak at approximately 620 nm, which may indicate the presence of hydrophilic porphyrins, (coproporphyrin) in OE19 cancer cells. Thus some cancers may have an additional emission peak. The 620 nm peak intensity increases in a time-dependent manner, concomitant with an increase at 636 nm, and this spectroscopic finding was supported by the HPLC-FL analysis conducted by NWH. In addition, the presence of the 620 nm was also linked to the appearance of a 680 nm peak. NWH and AMC observed different trends in the PpIX fluorescence ratio I_{620}/I_{636}, and at this stage it is unclear as to the cause of these differences. However, it was difficult to absolutely superimpose these results due to the alternative test set-ups used by each RTD partner. The presence and identity of the 620 nm peak, which changes in intensity with excitation wavelength, could indeed be a further avenue of research that could aid the detection and differentiation between cancerous and normal cell types.

The current versions of the Dynamic Blue prototypes, as an early cancer detection tool, were capable of detecting fluorescence differences between different types of cell lines (Refer to D4.2), and between non-dysplastic and dysplastic Barrett's tissue (Refer to D5.2). However, no significant inter-patient differences were found to distinguish between normal Barrett's epithelium from dysplastic tissue (Refer to D5.2). Thus, the Dynamic Blue device needs to be further optimised to increase any clinical discriminatory value.

11 Work carried out compared to work planned

All work carried out was in line with that to the work originally planned, with all tasks completed without any major revisions to the agreed description of work. Three versions of the Dynamic Blue device were received from 2M; each of which were slightly technically different, improving on the previous system, thus requiring addition testing and time. In addition to this, the staffing required for the project was not ready to start due to the quick start of the project. It took some time to arrange the necessary resources. The delay that this caused was rectified later in the project by putting more resources on the project.

This meant that several deliverables, for example 3.M1 (submitted M14 instead of M12); D4.M4 (submitted M17 instead of M12) and D4.1 (submitted M17 instead of M13) were submitted later than originally forecast. The later deliverables were all submitted on time.

The deliverable 5.M3 for the midterm report required the in-vitro analysis of biopsy samples obtained from 20 patients. AMC has significantly expanded on this task by performing ex-vivo spectroscopy on 26 sites in Barrett's esophagus EMR specimens (with corresponding biopsies) and 675 in-vivo spectroscopy measurements on 135 sites in Barrett's esophagus during real-time endoscopy. A total of 38 patients were investigated in this project. In addition, these tests used the first generation Dynamic Blue prototype with a multi wavelength laser set-up. The first part of WP5 therefore provided much more data and information than originally scheduled. First, data were largely collected in-vivo instead of the artificial in-vitro setting. Second, data were collected from a much larger group of patients than originally planned, thus increasing the statistical power and the extrinsic validity of the project. Third, data were obtained using the Dynamic Blue prototype which more closely resembles the actual clinical context of the final product. Fourth, the first phase allowed technical, methodological, and statistical preparation that are not only imperative for the second part of WP5 of the EDOCAL project but also allow technical adjustments of the prototype beneficial to the Dynamic Blue project.

In the original proposal, WP5 also mentioned basic research into the metabolites of porphyrin pathway and the measurements of differences in enzymes activity between different types of tissue. Although this might increase our basic understanding of 5-ALA fluorescence these results are not an absolute requirement for the development and validation of the laser system. In addition, these basic issues were also covered by the in-vitro studies performed by NWH. Based on the excellent results from NWH the aforementioned clinical studies and the fact that in-vivo spectroscopy studies result in the highest quality transferable knowledge for development and validation of the Dynamic Blue prototype it was decided that the AMC would shift the focus after some ex-vivo measurements towards in-vivo measurements.

During in-vivo measurements, the Dynamic Blue laser prototype set-up proved to be adequate for real-time clinical testing. The system set-up, combined with state-of-the-art endoscopic imaging technology allowed the measurement of high-quality auto-fluorescence spectra during real-time endoscopy in

Barrett's patients, thus closely resembling the actual clinical application of the final product of this project. This is a crucial step for testing and validating future prototypes.

A total of 2 x 11 patients with a Barrett's oesophagus (BO) were scheduled for a double imaging endoscopy (2 x 8 patients with BO and HGIN/EC and 2 x 3 patients with a non-dysplastic BO). During the endoscopy the BO would be imaged with the Dynamic Blue prototype pre- and post 5-ALA administration in a 30 mg/kg solution in orange juice, immediately followed by a correlating biopsy of the imaged areas. Patients are then brought to a recovery suite, where lights are dimmed and vitals are monitored for 3 hours, because of reported hypotension, nausea, vomiting and collapse. Due to the 5-ALA induced PpIX phototoxicity, patients are advised to stay out of direct (sun)light for at least 48 hours and keep out of the sun for a week.

5-ALA has been investigated in many clinical applications, and is most renowned in photodynamic therapy applications in dermatology and neurosurgery. At the moment, in Europe 5-ALA is only registered for use in patients by neurosurgeons and only after an extensive course and certification.

Availability of 5-ALA

The AMC approached multiple companies to obtain 5-ALA suitable for administration in patients, as described in the good manufacturing practice (GMP) guidelines. Medac GmbH is the only company currently producing GMP 5-ALA. Medac was contacted and a request was put in for GMP 5-ALA for oral administration. However, multiple aspects of our request hampered the decision of Medac to proceed with the production:

1. GMP 5-ALA is produced by Medac, based on the raw material as delivered by another company. The 5-ALA is registered under the brand name Gliolan, which is only available for neurosurgical purposes.
2. In order to supply GMP 5-ALA for patient administration in the GI tract, a specialized clinical trial batch would have to be prepared for the sole purpose of this study. This involves research and development costs, production costs and registration. Furthermore, legal council is required to investigate the legislative consequences of providing an experimental drug to the AMC for clinical studies, in terms of FDA/EMA approval. First shipment after legal approval would take at least 3 months.

Medac GmbH did not intend to pursue production of 5-ALA according to GMP guidelines for the sole purpose of the EDOCAL project. As a result of this the AMC proposed an alternative path to reach the results. The proposal was divided into 3 parts:

Part 1: in-vivo spectroscopy in 12 BO patients with HGIN/EC, without administration of 5-ALA. This will yield possible new insights on autofluorescence characteristics, which will always be present as 'background' fluorescence on which the PpIX fluorescence will be superimposed. Preliminary analysis of 233 autofluorescence spectra out of 53 patients, acquired in phase 1 of the EDOCAL project, suggests that a combination of multiple wavelenghts (mainly 365 and 405 nm) achieves high accuracy in distinguishing dysplastic from non-dysplastic Barrett's tissue. More data are required to validate these

results. In addition, the data have lead to the filing of two preliminary patents on spectroscopic diagnostic tools for dysplasia in BO.

Part 2: ex-vivo spectroscopy on cultivated specialized oesophageal cell-lines from Ninewells Hospital, Dundee before and after 5-ALA application. These cells are transplanted on a CAM model which allows for a simulated in-vivo situation with the advantages of a standardized ex-vivo setting. Moreover, by using NWH's cell lines, the consortium was responding to the feedback in the REA-report over the first period, in which a closer scientific relationship between NWH and AMC was advised.

Part 3: ex-vivo spectroscopy before and after 5-ALA application on biopsy specimens from 33 BO patients with HGIN/EC, transplanted on the CAM model, thus simulating a standardized in-vivo setting. The advantage of this transplanted biopsy model is 4 fold:

- No GMP 5-ALA would be required for administration. Non-GMP 5-ALA is easily available.
- The sample size can be increased from 22 to 33 patients. In phase 1 of the EDOCAL project 31 patients with HGIN/EC were included. Until now this number of patients was expanded to 53. Within these 53 patients 30 areas with HGIN/EC were identified, out of 223 measured locations. The original sample size of phase 2 (22 patients) therefore likely results in an inadequate statistical power. Therefore, by increasing the sample size to 33 patients, statistical power can be secured.
- The in-vivo CAM model allows a better exchange of results between the AMC and the in-vitro work of NWH. The results of both partners in the consortium could thus be better related.
- This in-vivo CAM model also allows easy testing of other photosensitizers in case 5-ALA proves to be an inaccurate photosensitizer.

CAM model

The proposed protocol uses a well-established in-vivo simulating model; the chorioallantoic membrane (CAM) model.

The CAM is the "placenta" in a fertilized chicken's egg. The CAM easily reached by cutting a window in the outer shell of the egg. The CAM is rich in blood vessels and as such can serve as a natural petri dish on which to grow cells, tissue blocks or even transplanted biopsies.

Many applications are known in the literature and the Department of Biomedical Engineering of the AMC has extensive experience with the CAM model. Tumour cell suspensions have been injected, as well as transplanted tumour spheroids – optimized tumour blocks – and biopsies. Also, multiple 5-ALA fluorescence studies have been performed with this model[1,2]. Therefore we feel confident that the CAM model will be a valuable, relevant model to execute this phase of the EDOCAL project.

The change proposal was submitted for approval and we were informed that no change to the DOW or other documents was required as we were not changing the targeted result of the project.

12 Efforts to involve others

The EDOCAL project is a collaboration between 3 SME's, developing innovative medical technology, and 2 RTD's, evaluating the technology.

The AMC performed multiple experiments, as described in chapters 1 and 2. The experiments were performed on the department of gastroenterology and hepatology, in close collaboration with the department of biomedical engineering and physics. Furthermore, the AMC worked closely together with the other RTD partner of the EDOCAL project; NWH. For the technical development and evaluation of the prototype, a strong relationship with 2M and TopGAN was established. The optical fiber probe was developed in collaboration with CeramOptec (Germany).

For the evaluation of the tissue specimens used for the experiments, the expertise of the department of pathology was highly valuable. A standardized protocol was developed to assess all tissue, whether derived from biopsy specimens, resection specimens, or the CAM model.

At the AMC, the departments of industrial engineering, experimental biomedical engineering and animal affairs were involved in the development of the CAM model.

The SMEs worked with the University of Delft on the design and required fabrication processes for the tunable laser.

The SMEs also worked with various packaging companies in the UK and Germany in order to develop a suitable packaging for the lasers.

2M has worked with ASML (leading wafer stepper company) to discuss the technology that is necessary for the wafer steppers to be able to process the Dynamic Blue lasers in the future.

NWH have been sharing their results with Prof. Lorenzo Brancaleon of the University of Texas, who is an expert in Photobiology.

There have been contacts with the University of Seoul, South Korea on the geometry of the Photonic Crystals for the laser design.

2M has had contacts with MIT on simulating the use of the Photonic Crystals for the laser.

2M has involved a number of students from the Louis Pasteur Institute in Strasbourg for Photonic Crystal design studies.

13 Summary of results and conclusions

The results of the EDOCAL project can be summarized as follows:

- A tuneable blue laser system prototype was defined and built for research purposes.
- The test system was capable of delivering and detecting PpIX fluorescence in solution and in cell-lines (i.e. the lasers had sufficient power)
- The test system was adequate (i.e. laser power was sufficient) and in-vivo and ex-vivo fluorescence spectroscopy was successfully achieved.
- Experiments to detect cancer using shifts in excitation and emission wavelengths were carried out in 70 patients and we obtained a total of 1557 spectra from 290 sites in the oesophagus. Using the prototype, each site was illuminated with multiple wavelengths followed by registration of the corresponding auto-fluorescence spectrum and tissue biopsy for histological correlation.
- Results from the EDOCAL prototype show that it is possible to discriminate early cancer with 80% sensitivity and 81% specificity , which meets the performance requirements targeted at the start of the EDOCAL project.
- The combination of 365nm and 405nm excitation improves the sensitivity from 67% up to 100%.
- Proof of concept has been achieved for early detection of cancer based on a tuneable blue laser technology.
- As the endogenous fluorescence emission reflects the nature of the fluorophore, the combination of AF peak maxima, intensity and PpIX peak maxima and intensity may be a powerful tool in differentiating tissue staging in cancer and other diseases.
- The most efficient excitation wavelengths to discriminate non-dysplastic from dysplastic Barrett's tissue are 410nm and 416nm.
- An additional emission peak at 620nm was observed and could potentially be used to further improve the discrimination of different tissue types.
- The optimal excitation wavelength differs for non-dysplastic and dysplastic Barrett's tissue in 45% of cases. This supports the hypothesis that the shift in excitation wavelength may have diagnostic value.
- Preliminary specifications for a first demonstrator are completed.
- Two patent applications for Laser excited fluorescence using combinations of laser wavelengths to contrast the boundaries of healthy and cancerous tissue and cancer detection and diagnosis using laser fluorescence have been filed.

- This shift in optimal excitation wavelength was independent from the tissue type, which may limit its clinical impact. How such a peak shift may be utilized in the clinical detection of dysplasia using 5-ALA depends on the further development of the Dynamic Blue prototype and detection algorithms.
- The performance of the prototype in combination with the photo-sensitizer used in the EDOCAL project is still suboptimal and requires further optimization, before results can be translated into patient-related diagnostic value.