IAEA-TECDOC-1562

Developing a Programme on Molecular Nuclear Medicine

Proceedings of a technical meeting held in Vienna, 29 November–1 December 2004

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FOREWORD

During the last decades, new methodologies have emerged in the molecular nuclear medicine field developed to contribute to the detection, diagnosis, staging and treatment follow-up of human diseases. Single photon emission computed tomography (SPECT) and positron emission tomography (PET) are two examples of this methodology that enabled the study of molecular alterations of cell metabolism in the living subject with non-invasive approaches. 18-fluorine fluorodeoxyglucose positron emission tomography (FDG-PET) is used for many disease diagnoses, differential diagnosis and treatment follow-up. Like FDG, new molecules are also being identified and are promising candidates to be used.

Molecular imaging studies the expression of genes involved in the evolution of different diseases. This data has been shown to be a reliable prognostic marker, for accurate diagnosis or for predicting response to treatment in certain cases. The use of molecular imaging in the evaluation of exogenous gene therapy and the study of endogenous gene expression in genetic, neurological, cardiovascular and neoplastic diseases will be of significant importance worldwide in the near future.

The use of nuclear medicine and molecular imaging for the study of a disease assures the determination of integral parameters for prognosis and diagnosis. The improvement of the therapeutic decisions involved with the stage and prognosis of a disease will certainly add to the clinical studies that are designed for patient care, treatment and survival improvement. Many efforts have been made and will continue in the future to demonstrate the potential of the association of molecular nuclear technology and nuclear medicine imaging, since it has been shown to be useful and applicable to many important diseases.

In addition, molecular biology techniques, such as polymerase chain reaction (PCR) and differential gene expression have added important findings to the study of disease pathogenesis. These techniques have been increasingly applied for revealing the different profiles of normal and affected cells or tissues and also for the following-up treatment of certain diseases like minimal residual disease (MRD). The detection of changes in the level of transcription of certain genes using this approach has been a useful tool for the early detection of disease, improving the patient survival.

At the Technical Meeting on Developing a Medium to Long Term Programme on Molecular Nuclear Medicine, held in Vienna, 29 November–1 December 2004, certain areas were selected as the best candidates to be included in the IAEA's programme in relation to their applicability and potential to improve human health. The IAEA will continue supporting training activities and fellowships, and encouraging developing Member States to take advantage of the use of isotopic advanced molecular techniques for the resolution of their health problems.

This TECDOC contains useful information for health workers in the nuclear medicine and molecular biology fields. Previous IAEA publications, Nuclear Medicine Resources Manual (STI/PUB/1198), Radionuclides in Molecular Technology for Diagnosis of Communicable Diseases (IAEA-TECDOC-748), In Vitro Radionuclide Techniques in Medical Diagnosis (IAEA-TECDOC-1001) and Organization of a Radioisotope Based Molecular Biology Laboratory (IAEA-TECDOC-1528), are separately focused on nuclear medicine and molecular biology techniques applied to human diseases, while the present publication provides presentations on the advances of molecular nuclear medicine techniques.

The IAEA wishes to thank all participants of the meeting for their contribution to this publication. The IAEA officer responsible for this publication was B. Khan of the Division of Human Health.

EDITORIAL NOTE

CONTENTS

SUMMARY

1. Introduction

The main objective of this meeting was to define strategies to combine molecular biology techniques with nuclear medicine imaging in the activities to be developed in the next five years in Member States.

Two objectives could be identified:

(a) Short to medium term objective

Linking the images from nuclear medicine procedures with results obtained from molecular biology techniques to obtain better parameters for prognosis and diagnosis of human diseases

(b) Long term objective

Development and evaluation of radioactive biomolecules using results obtained with molecular biology techniques.

The focus of the proposed projects that can be found herein is on objective (a). Objective (b), besides being considered a long term goal, is beyond the remit of the type of research being undertaken in this field at the IAEA and is hindered by budget constraints, involving not only research but development as well (biotechnology and radiopharmacy).

2. Identifying the needs

The major areas in medicine that should have a strategic focus are:

- Communicable diseases

The use of molecular markers and inflammation/infection imaging applied to communicable diseases has evolved dramatically during the past twenty years. Deoxyribonucleic acid (DNA) amplification and molecular hybridization with radiolabelled probes are molecular nuclear methods commonly used to address problems related to communicable diseases. Proteomic research has been developed recently as a valuable source of information about all processes related to the life and metabolism of infectious microorganisms due to the universal involvement of proteins in these mechanisms.

- Malaria – detection of asymptomatic infection and drug resistance
- Human Immunodeficiency Virus (HIV) – genetic diversity, vaccine development, opportunistic infections, concomitant cancers (mostly lymphoma), differentiating malignancies from infections in the central nervous system (toxoplasmosis and Chagas disease)
- Tuberculosis – drug resistance, transmission patterns, direct molecular diagnosis and typing of the strains in sputum, detection of extrapulmonary tuberculosis
- Dengue – genetic diversity, vaccine development, defining risk factors for hemorrhagic dengue (pathogenesis)
- Hepatitis C – genotyping, drug resistance, cancer association
- Leishmaniasis – drug development, vaccine targets, pathogenesis
- *Helicobacter pylori* – diagnosis, cancer association

- Cancer

Nuclear medicine techniques, like 18-fluorine fluoro deoxyglucose- positron emission tomography (FDG-PET), are being used and accepted as important methods for staging and evaluating therapy response of many tumours. Also polymerase chain reaction (PCR) amplification and sequencing of affected genes, detection of molecular markers of prognosis and recurrence, are useful tools applied to cancer treatment and follow-up.

- Lymphoma and leukaemia – prognosis and staging, detection of minimal residual disease (MRD), therapeutic response
- Hepatocellular carcinoma – detection of recurrence after therapy, response to treatment, detection of multiple foci.
- Breast cancer – molecular markers and genetic counselling, study of sentinel lymph node, staging, tumour recurrence, therapy planning.
- Cervical cancer – association with human papillomavirus (HPV) infection, staging and therapeutic response-detection of recurrence
- Thyroid cancer – prognostic molecular markers, detection and treatment of iodine negative lesions
- Oesophageal and gastric cancer – staging, recurrence, assessment of prognosis and treatment planning, association with *Helicobacter pylori*
- Lung cancer – differential diagnosis, staging, distant metastasis, therapy monitoring, treatment planning for chemotherapy or radiotherapy
- Soft tissue sarcomas and osteosarcoma – differential diagnosis, metastasis localization and treatment planning.
- Colon cancer – molecular markers, recurrence, metastasis, therapy monitoring
- Prostate cancer – detection of tumours in patients with rising prostate specific antigen (PSA) levels, MRD, recurrence, lymph node involvement, bone metastasis, molecular markers

- Neurological diseases

FDG-PET, ^{99m}Tc-HMPAO (technetium-99m-hexamethylpropyleneamine oxime) or ^{99m}Tc-ECD (technetium-99m-ethyl cysteinate dimmer) are examples of nuclear medicine techniques that can be used in the study of neurological diseases when differential diagnosis must be resolved to decide a specific treatment and prognosis.

- Dementia and Alzheimer disease – differential diagnosis between depressions and dementia, therapeutic response, molecular marker
- Parkinson disease – differential diagnosis with multi-system degeneration
- Epilepsy – focus identification

- Cardiovascular diseases

The high resolution of PET has been an advantage over other techniques for the study of cardiac diseases such as coronary artery disease. Also, combined with ^{13}N-ammonia it can be used for the evaluation of myocardial functional recovery of viable myocardium.

- Atherosclerosis – identification of active plaques
- Cardiovascular effects of diabetes – identification of alterations in perfusion
- Cerebrovascular accident – diagnosis and identification of extent, early treatment planning, prognosis, therapeutic response
- Hypertension - molecular markers, association as a pathogenic factor to other diseases

- Genetic diseases

Genetic diseases have benefited widely from the use of molecular techniques like the sequencing of affected genes involved in the development of the disease and the early detection of affected individuals.

- Myotonic dystrophies – molecular markers and genetic counselling
- Cystic fibrosis - molecular markers and genetic counselling, therapy evaluation and extent of disease affection, early detection of lung infection
- Huntington disease – molecular markers, early detection

Based on the participants' experience and knowledge, a summary of the molecular biology and imaging techniques that can be employed to study human diseases is reported in Table I. These diseases can therefore be the objects of future work. Table II summarizes in which of the diseases reported in Table I both molecular biology and imaging techniques are available, as the combined use of in-vitro and in-vivo molecular techniques is one of the meeting's goal.

In order to optimize the activities of the consultants, some of the above projects were chosen to be developed. In consensus with the main objective of the IAEA, the group decided to focus mainly on oncology. The diseases on which future IAEA projects should focus are summarized in Table III. Nevertheless, the other areas were also contemplated with one specific project per segment. Diseases with very low prevalence and a benign course should not be the focus of the future activities (iodine negative lesion –thyroid cancer– was not included).

3. Summary for future activities

(a) Tuberculosis – Diagnosis of extrapulmonary tuberculosis – molecular diagnosis is available for tuberculosis using PCR coupled to radiolabelled probes enhancing the sensitivity of the detection with a more robust method and cost effective approach. The clinical problem encountered in this disease is to get a correct diagnosis in patients with a history of tuberculosis or a positive skin test (PPD positive) together with fever of unknown origin (at least 21 days). In this scenario PET-FDG is capable of identifying the inflammation site and directing specific diagnosis. Alternatively, a ^{67}Ga scan can also be informative when PET is not available. These samples should also be submitted to conventional diagnosis such as histopathology and culture using ^{14}C medium that provides results 15 days faster than the traditional Lowenstein Jensen medium. A diagnostic algorithm should be developed combining molecular and nuclear medicine techniques.

Table I. Available techniques to address the identified human diseases

Disease	Molecular markers			Molecular imaging					Recommendations*
	Diagnosis	Prognosis and/or drug resistance	Differential diagnosis	Staging	Recurrence	Prognosis	Response to treatment	Early detection	
Communicable diseases									
Malaria	+	+							
HIV	+	+	+						+
Tuberculosis	+	+			Detection of extrapulmonary tuberculosis				+
Dengue	+								
Hepatitis C	+	+							
Leishmaniasis	+								
Helicobacter pylori	+								
Cancer									
Lymphoma	+	+		+	+	+	+		+
Leukemia	+	+							
Hepatocarcinoma		+		+	+		+		
Breast cancer	+	+		+	+				+
Cervical cancer	+	+		+	+		+		+
Thyroid cancer	+	+			Detection of iodine negative lesions				+
Oesophageal and gastric cancer		+		+	+	+			
Lung cancer		+	+	+			+		+
Soft tissue sarcomas and osteosarcoma		+	+	+					
Colon cancer	+	+		+	+		+		+
Prostate cancer	+	+		+	+				+
Neurological diseases									
Dementia and Alzheimer disease	+	+	+				+		+
Epilepsy					Focus identification				
Parkinson disease			+						
Cardiovascular diseases									
Atherosclerosis	+	+			Detection of active plaques				+
Cerebrovascular accident			Diagnosis and extent			+	+		

Table I. Available techniques to address the identified human diseases (continues)

Disease	Molecular markers		Molecular imaging						Recommendations*
	Diagnosis	Prognosis and/or drug resistance	Differential diagnosis	Staging	Recurrence	Prognosis	Response to treatment	Early detection	
Cardiovascular effects of diabetes					Perfusion alterations				
Hypertension	+	+							
Genetic diseases									
Myotonic dystrophies	+	+							
Cystic fibrosis	+	+			Extent, early detection of lung infection				+
Huntington disease	+	+						+	+

*Recommendations mean that the proposed techniques could be implemented in future activities.
+ Means that there are techniques available for the purpose related to the column and the disease related to the row.

Table II. Summary of the diseases listed in Table I that present the potential to be addressed by both approaches.

Disease	Molecular markers		Molecular imaging	Recommendations*
	Diagnosis	Prognosis and/or drug resistance		
Communicable diseases				
1.HIV	+	+	+	+
2. Tuberculosis	+	+	+	+
Cancer				
3. Lymphoma	+	+	+	+
4. Breast cancer	+	+	+	+
5. Cervical cancer	+	+	+	+
6. Thyroid cancer	+	+	+	+
7. Lung cancer		+	+	+
8. Colon cancer	+	+	+	+
9. Prostate cancer	+	+	+	+
Neurological diseases				
10. Dementia and Alzheimer disease				+
Cardiovascular disease				
11. Atherosclerosis	+	+	+	+
Genetic diseases				
12. Cystic fibrosis	+	+	+	+
13. Huntington disease	+	+	+	+

*Recommendations mean that the proposed techniques could be implemented in future activities.
+ Means that there are techniques available for the purpose related to the column and the disease related to the row.

Table III. Shortlist of diseases for development of future activities.

Disease	Molecular markers		Molecular imaging	Recommendations*
	Diagnosis	Prognosis and/or drug resistance		
A. Tuberculosis	+	+	+	+
B. Lymphoma	+	+	+	+
C. Breast cancer	+	+	+	+
D. Colon cancer	+	+	+	+
E. Prostate cancer	+	+	+	+
F. Dementia and Alzheimer	+	+	+	+
G. Atherosclerosis	+	+	+	+
H. Cystic fibrosis	+	+	+	+

*Recommendations mean that the proposed techniques could be implemented in future activities.
+ Means that there are techniques available for the purpose related to the column and the disease related to the row.

(b) Lymphoma – Diffuse large B-cell Lymphoma (DLBL; "High-Grade NHL - Non Hodgkin Lymphoma") is rapidly fatal without treatment; for up to half of all patients it is curable with well-established combination chemotherapy regimens. During the past 3 years gene expression profiling, using DNA microarrays, has demonstrated that lymphomas expressing certain combinations of activated genes are associated with a good outcome (LMO2, BCL6, FN1), while other gene expression signatures are associated with a poor prognosis (BCL2, SCYA3, CCND2). This issue can be addressed by Northern blots with radiolabelled probes. FDG-PET can be used for staging the disease, evaluating the response to therapy and identifying the eventual relapse. Alternatively, ^{67}Ga scan can also be informative when PET is not available. The FDG accumulation, prior and after treatment, may be used in combination with molecular markers to evaluate prognosis and consequently for the decision to apply more aggressive treatment schemes.

(c) Breast cancer – Sentinel lymph node biopsy (SLNB) is a method applied for detecting invasions of neoplastic cells in the first node receiving lymphatic drainage. SLN has been studied in breast tumours and the technique proved to be useful. Sentinel node biopsy, in breast cancer, is a promising technique for predicting histological findings in the remaining axillary lymph nodes, in patients with clinically node-negative breast cancer. After identifying the node, laboratory techniques must be applied in order to evaluate the presence of neoplastic cells. Pathological analysis (immunohistochemistry analysis) using monoclonal antibodies has brought sensitivity and specificity to this task. Molecular biology techniques can be used to enhance the sensitivity of the detection of micrometastasis. More advanced disease imaging techniques such as FDG-PET are useful for staging, radiotherapy planning and detection of recurrence or metastases in patients with rising tumour markers. As part of combined treatment schedules involving antiangiogenic drugs, scintigraphic information about tumour angiogenesis e.g. by use of radiolabelled arginine-glycine-aspartate (RGD) peptides, may be useful. For prognostic purposes, presence of mutations in the p53 gene, expression of epidermal growth factor (EGF) receptor (Her-2 and ERBB-2) and matrix metalloproteinases (MMPs) should be assessed in the tumour cells. As in lymphoma, these molecular markers could be combined with non-invasive imaging techniques.

(d) Colon cancer – The main problems in colon cancer are local control and control of distant metastases. The first problem is addressed by surgery followed by radiation therapy. However, problems arise when the patient suffers from pain and shows an undefined mass in computed tomography (CT) after treatment. Here, the differentiation between scar and tumour recurrence is important. This can be addressed by the use of FDG-PET. The other problem is therapy monitoring of liver metastases. The standard treatment is administration of fluorouracil (5-FU) together with folic acid. However, only 10-15 % of the patients are responders. In order to identify non-responders that may profit from change of treatment at an early stage, monitoring techniques are needed. This can be done using FDG-PET. A third problem is the patient with a history of colon cancer and rising carcinoembryonic antigen (CEA) levels. Identification of tumour metastases or local recurrence can be done with FDG-PET prior to and after the first cycle of chemotherapy. For better biological characterization, analysis of surgical specimens for molecular markers, such as mutations in the p53, APC and K-RAS genes, should be searched. Since there is evidence for an association of RAS mutation and glucose transport, this relationship may be studied in these tumours.

(e) Prostate cancer – In older patients with rising PSA levels the detection of prostate carcinoma is necessary to initiate treatment at an early stage of disease. Since most of these patients may suffer from hyperplasia, malignant lesions must be differentiated from benign disease. Due to a low glucose consumption in most of these tumours (except tumours with a

very high Gleason score) FDG-PET cannot be used. As an alternative ^{11}C-acetate, ^{11}C-choline, ^{18}F-choline, ^{18}F-fluoroethylcholine can be used. Compounds based on ^{18}F are preferred because they can be distributed from production centres (satellite concept). The same holds true for the detection of tumour recurrence in patients after surgery and with rising PSA levels. In that situation PET, as well as bone scintigraphy is applied. For proper treatment planning, especially in the case of radiation therapy, information about the local regional lymph node involvement is needed. Cat-like, a calcium channel molecule and cancer-testis antigens (CTAs), (MAGE, PRAME) are over expressed in tumours with a higher Gleason score. Therefore, the evaluation of the over expression of these genes is a prognostic marker that should be followed. Since prostate carcinomas are expected to express somatostatin receptors and receptors of the bombesin family, correlative studies of receptor imaging and molecular biology analysis of these tumours are possible.

(f) Dementia and Alzheimer Disease – Patients presenting cognitive impairment may suffer from a variety of neurologic and psychiatric disorders. Since the several underlying pathologies differ significantly in treatment and prognosis there is a need for individual diagnosis and optimization of therapy. The most common applied method is FDG-PET. However, also perfusion measurements with ^{99m}Tc-HMPAO of ^{99m}Tc-ECD can be used. Tracers for the visualization of amyloid plaque formation are under development and could be available in the near future and may be related to the findings of molecular biology techniques. As molecular markers, polymorphisms in the ApoE gene can be studied.

(g) Atherosclerosis – The urgent clinical problem in this disease is the identification of vulnerable plaques that ultimately lead to infarction of the brain and the heart. Molecular imaging techniques could visualize these plaques in patients before infarction occurs, leading to early treatment. An available procedure is FDG-PET which is based on inflammatory reactions in vulnerable plaques causing increased tracer uptake as has been shown in carotid disease, diffuse vasculitis and coronary artery disease. Fusion images can improve diagnostic accuracy. This can be done either using a PET-CT system or a purpose designed software when separate imaging devices generate the images. Alternative targets are apoptosis and matrix metalloproteinases that can be visualized by radiolabelled annexin V or MCP-1, with the support of a dedicated radiopharmacy. Molecular markers of medical interest are also matrix metalloproteinases. With respect to angiogenesis in heart infarction, molecular markers for angiogenesis and nuclear medicine procedures for perfusion imaging such as ^{82}Rb-PET, $^{13}NH_3$-PET, ^{99m}Tc-Tetrafosmine or ^{99m}Tc-MIBI could be combined to assess the extent of the disease.

(h) Cystic fibrosis - Cystic fibrosis (CF), a worldwide disease occurring in most ethnic groups, is a generalized exocrine disorder characterized by highly variable clinical presentation. Both severity of disease and rate of progression of CF show an extreme heterogeneity; some of these variations may result from the type of mutation on the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The high degree of genetic heterogeneity for the CFTR mutations has been observed in several population groups. The deletion of the phenylalanine residue at the position 508 of the CFTR protein is the most common mutation in Caucasians leading to the highest morbidity. However, it is known that in several other ethnic groups, affected chromosomes present different mutations. In the absence of complete mutation definition in several ethnic groups in the world, additional methods must be applied in order to define other mutations in CFTR beyond the most commonly found in Caucasian population in patients with pancreatic dysfunction and pulmonary infections. Perfusion ventilation studies can assess the pulmonary problem of cystic fibrosis patients providing data for the better management of the affected children, and

radionuclide imaging can be used to detect pulmonary infections in these patients. Association of different mutations with impairment of pulmonary or biliary and pancreatic functions assessed by molecular imaging techniques can be investigated.

4. Minimum requirements to participate in future activities

- A nuclear medicine facility with at least one gamma camera and preferably a PET scanner.
- A quality control system for the instrumentation should be in place, including environmental assessment for the equipment. It is recommended to implement an external quality control system including a cross calibration of PET scanners through inter-laboratory sharing of calibration devices.
- An established molecular biology laboratory performing most of the common techniques should be in place and preferably in the same institution as the nuclear medicine facility. The personnel of both sets should work as the team defining the best logistics for getting and processing the samples in a proper way.
- Radiation safety instructions must be written following the format of a manual for the molecular biology laboratory.
- A license for the use of radionuclides must be obtained with the government in every case.
- A radiopharmaceutical laboratory must be in place to generate the necessary tracers and to maintain quality control.
- The sample size of the study must be obtained during the timeframe of the work. Therefore the patients must be made available by the chosen centre.
- Suitable qualified personnel in both technical aspects (nuclear medicine and molecular biology) should be involved for the study.

5. Recommendations

- A database containing information about the centres with available nuclear medicine facilities including PET should be developed.
- The studies are based on enrolment of patients and diagnostic and follow up procedures. An epidemiological and statistical analysis of every activity is mandatory before the implementation of the work so that a proper sample size can be calculated.
- A quality control programme should be in place before the onset of the activities including written standard operational procedures and protocols.

Medical imaging in the new century

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Abstract. This paper is a brief introduction on nuclear molecular imaging, dealing with the state of medical imaging for what concerns radionuclide imaging. After a description of the available nuclear technologies and their characteristics, the new developments in this field are illustrated, with particular attention to Positron emission tomography and its possible application in future gene therapy. Existing clinical application for the diagnosis of most diseases is illustrated, defining the role of the most diffuse radiopharmaceutical, the glucose analogue fluoro-deoxy-glucose. This radiopharmaceutical is likely to be the first to be introduced in developing countries, due to its biological properties and to its relative ease of production.

1. Introduction

Medical imaging involves a broad spectrum of methods that are able to visualize anatomical and functional alterations in the living tissues. It is well known that functional alterations precede morphological ones and that the deeper the level of imaging which is available, the earlier the disease can be detected and, hopefully, cured. This is particularly true when dealing with cancer. Radiological imaging using X rays, particularly multi-slice computed tomography scanners, is nowadays able to see small anatomical lesions, below 1 mm in size. The same concept is true for ultrasound imaging, which is able to give incomparable spatial resolution when the probe can be positioned in close contact with the organ or apparatus to be studied. Magnetic resonance is considered a morphological and functional imaging method, since its detailed images can also give some functional data when using spectroscopy analysis.

Nuclear medicine techniques, like single photon emission computed tomography (SPECT) and positron emission tomography (PET), are able to visualize molecular alterations in the living subject. The small size of the used radio-pharmaceuticals allows non-interference with the metabolic processes while visualizing them. This is a dramatic advantage over any other imaging method, which most often requires a contrast media to be injected in order to get images with high contrast resolution. Nuclear medicine imaging, particularly PET, can measure cell metabolism, transmitter synthesis, enzyme activity, protein production, tissue viability and many others. Any alterations in these metabolic pathways can be seen as well, allowing an early diagnosis and treatment of the disease. Early evaluation of the disease leads to early evaluation of therapy efficacy. In modern oncology, where expensive drugs can have high efficacy in selected populations, it is of paramount importance to evaluate as early as possible if a patient is or not a responder to a specific therapy. This information precedes morphological changes by weeks or months and allows tailoring the best therapy for each patient in a short time, avoiding the possible side effects of a useless therapy.

Radionuclide molecular imaging techniques are expected to be able to detect diseases in a very early phase, before any onset of symptoms. In this view, the question is when the patient has to go for a check-up using these techniques. The answer is based on the knowledge of familiar and genetics characteristics, living and working environment and habits of any single individual, which is the key to stratify the patient's risk for a specific disease.

If this approach proves to be successful, other consideration must be taken into account, related to exposure of the population to ionizing radiation and to the costs of implementing these expensive technologies on a large scale, such as is needed for screening procedures.

Clinical multimodality imaging has been proposed to overcome the lack of anatomical landmarks, which characterises nuclear medicine techniques. This method consists of the registration and fusion of tomographic images acquired in different modalities, like PET, SPECT, CT (computed tomography) and MRI (magnetic resonance imaging). Dedicated acquisition protocols and image software are required to successfully create multimodality image, but the results are of high quality. Combined PET/CT and SPECT/CT scanners are commercially available and PET/CT is quite diffuse in the EU and USA. These scanners are able to acquire PET and CT in the same patient in a short time, and can create and display fusion images in a very short and effective way. This approach has been demonstrated to increase the diagnostic accuracy of stand-alone PET scanners. Figures 1 and 2 are examples of multimodality medical images.

2. Radionuclide imaging techniques

Positron emission tomography (PET) is the leading method in nuclear molecular imaging. Its principle is based on the possibility to label biological molecules with a positron emitting isotope. These radionuclides emit a positron from their nucleus that has a high probability to annihilate with an electron in a short time. The annihilation gives way to the production of two 511 KeV photons which travel at 180 degrees apart. These “coincidence” photons can be detected using dedicated scanners, which have particular crystals for the photon detection and electronic circuits to localize the emissions in time and space co-ordinates. The most frequently used isotopes in PET are oxygen-15, nitrogen-13, carbon-11 and fluorine-18 that are mostly used as a hydrogen substitute. Other isotopes, such as gallium-68, rubidium-82, iodine-124, copper-60 and copper-64, can be used as well. The production of such radionuclides relies mostly on the availability of a cyclotron dedicated to clinical activity. Some isotopes can be delivered to satellite centres or produced using a generator, allowing the labelling of some molecules also where a cyclotron is not available.

The radiopharmaceuticals are synthesized in dedicated radiochemical laboratories and injected into the patients using a venous access. The radiopharmaceuticals, also called tracers, act as molecular probes and, thanks to the radiolabelling, it is possible to follow their distribution and concentration throughout the body.

As described in the previous section, PET images can be easily registered and fused using a PET/CT scanner. Figure 3 schematically illustrates how the PET and the CT gantries are joined together in a single body.

Single photon emission computed tomography (SPECT) can also be used for molecular imaging, but this technique requires a different type of scanner, called gamma camera. The biological principle of SPECT is exactly the same of PET, the only difference being the use of gamma-emitting radioisotopes. SPECT has some major physical disadvantages when compared to PET, related to the detection instrument used: lower spatial

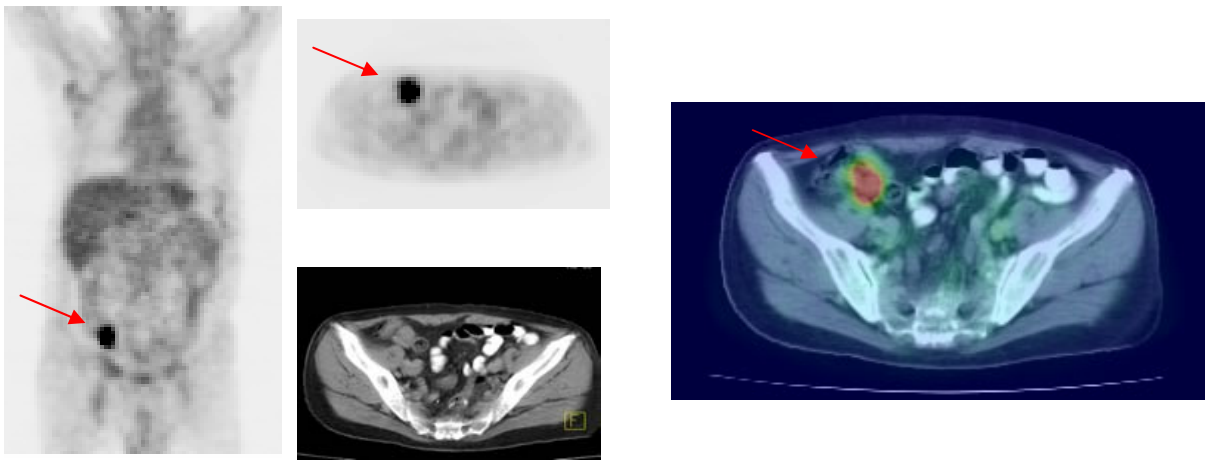

Figure 1. PET/CT multimodality images. The left panel shows a coronal view of an attenuation corrected total-body FDG image. The patient had a suspected relapse of colon cancer for an increasing CEA value, but the CT scan was reported as negative (lower image mid-panel). The PET image clearly shows a pelvic relapse (upper image mid-panel) which can be localized in the PET/CT fused images (right panel).

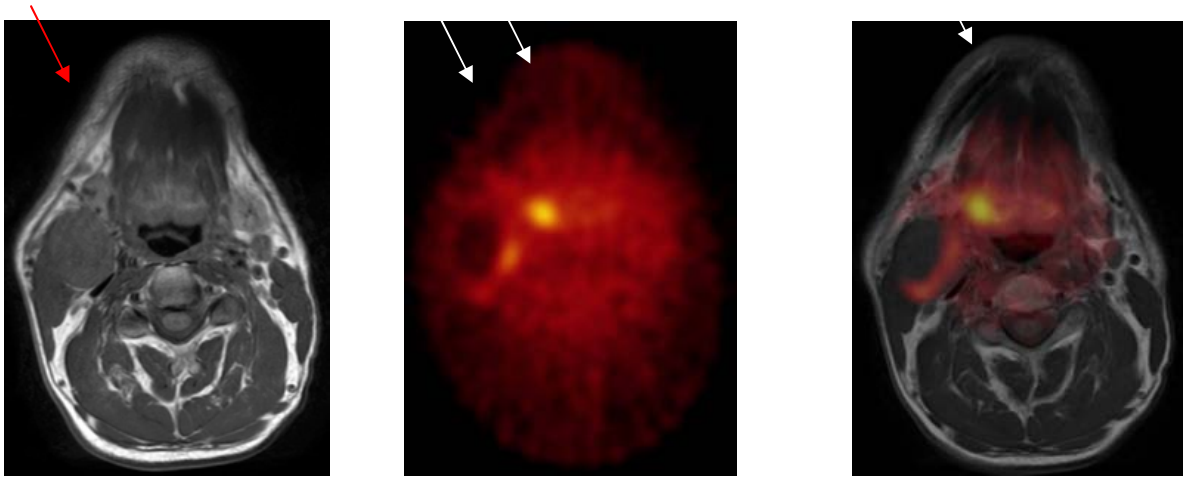

Figure 2. PET/MR multimodality images. The left panel illustrated an axial T2 weighted MR image which shows an enlarged left cervical node (third level) without any clear evidence of the primary tumour The mid-panel shows the corresponding FDG PET axial slice, which clearly shows the necrotic node and the primary tumour, which can be localised in the left tonsil of the tongue on fused images (right panel).

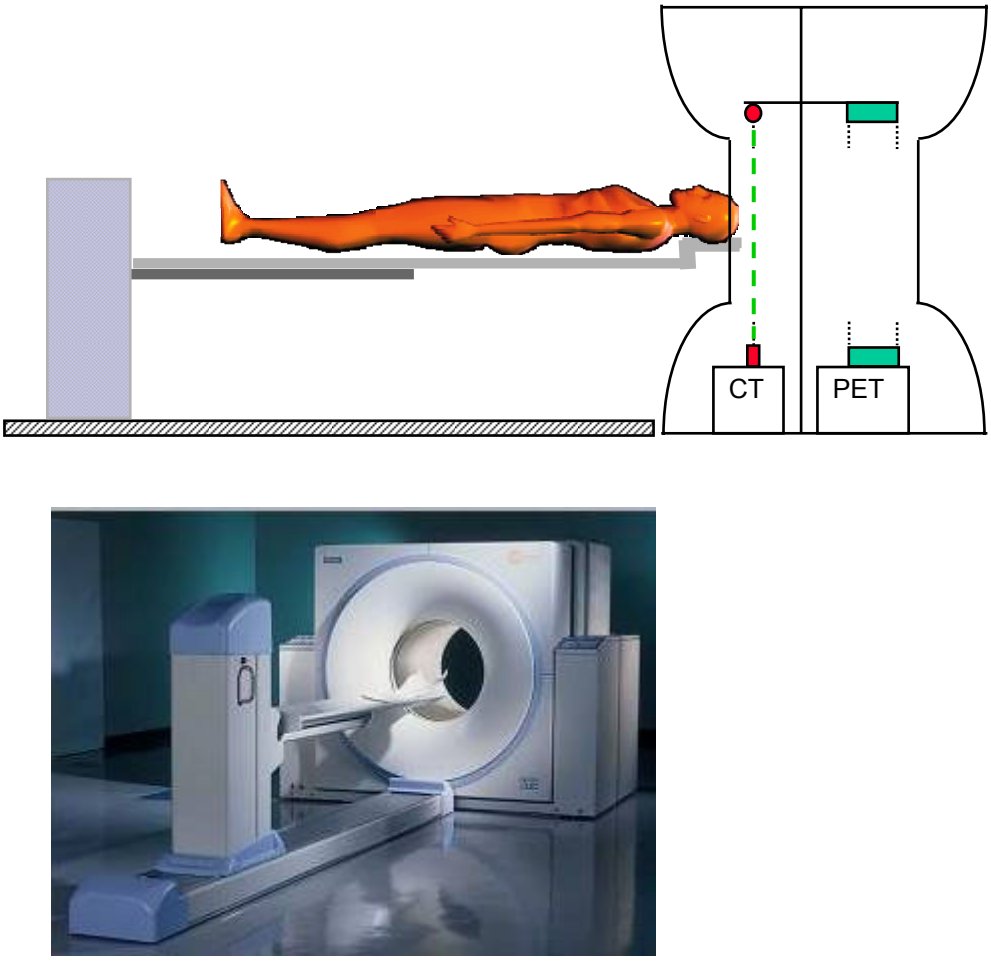

Figure 3. Schematic diagram of a PET/CT scanner. Note the two separate gantries joined in a single shell. Images are separately acquired but are registered and fused immediately, "on the fly". The photo shows a typical clinical PET/CT scanner. Images courtesy of Siemens Medical Solutions, Erlangen (D).

and temporal resolution and the need for a geometric collimation of the emitted photons, which reduces the sensitivity and makes quantification more difficult. While PET sensitivity is quite high, in the order of $10^{-11} – 10^{-12}$ mole/L, sensitivity of SPECT is 15 times less using the clinical system, but can be improved using special collimators called "pinhole".

From a radiopharmaceutical point of view, it must be stressed that positron emitting radionuclides can easily substitute naturally occurring atoms in biological molecules, while SPECT radiopharmaceuticals almost always need a carrier for the radionuclide to be linked with the biological molecule. This results in a final molecule that can be very different from the biological probe. On the other hand, SPECT allows the simultaneous detection of more than one probe, because it is possible to use different radionuclides with different photon energies (i.e. technetium-99m and indium-111), while all PET radiopharmaceuticals emit the same energy photons and cannot be distinguished when administered at the same time. A method to overcome this limit is to use very short half-life isotopes, such as oxygen-15, in order to get images from the probes at very short time intervals.

The development of small animal scanners is of paramount importance for molecular imaging, not only for nuclear medicine techniques. New generation scanners are expected to have a spatial resolution very close to 1 mm. This approach is particularly useful to validate new radiopharmaceuticals, before their clinical use and to radiolabel new drugs and check their in-vivo bio-distribution in the living animal. Moreover, PET/CT systems for small animals are already available, allowing overcoming the lack of anatomical landmarks which limit the ability to locate biological events when using stand alone PET systems.

3. Molecular imaging strategies

In vivo molecular imaging with PET could play an important role in pre-clinical and clinical research, particularly for gene therapy. In fact, the visualization of gene expression will have an important impact on the development of this new research field. The appropriate combination of PET reporter gene (PRG) and PET reporter probe (PRP) will be able to provide qualitative and quantitative information used to define parameters that are essential for the proper understanding of the gene therapy process. A PRG should be a gene whose product can be detected using a PRP. This means the PRG should be expressed only in defined tissues or organs and should be able to induce the cell to produce a receptor, an enzyme or a transporter that causes no damages or alterations in the modified cell. On the other side, the PRP should only accumulate in target tissues with the highest specificity.

The imaging of molecular processes has been divided in three main categories. Direct molecular imaging has been defined as a probe-target interaction, the resulting image being directly related to the interaction between probe and target. Indirect imaging involves multiple components and has major representation in the PRG-PRP interaction. Surrogate imaging is already feasible with existing radiopharmaceuticals; its limitation is related to the fact that it reflects the interaction between one or more endogenous molecular process.

One of the most used PRG is the Herpes Simplex type 1 virus thymidine kinase (HSV1-tk), which has been used in many clinical trials. Radiolabelled uracil nucleoside and acycloguanosine derivatives have been already used as experimental PRP in humans: ^{124}I-iodo-fluoro-deoxy-arabinofuranosyl-iodouracil (FIAU) and ^{18}F-fluoro-hydroxymethylbutyl-guanine (FHBG) are two examples of radiopharmaceuticals that can be successfully employed to visualize HSV-tk activity, FIAU being demonstrated to be the more efficient probe among the two.

Other examples of PRG-PRP systems are dopamine D2 receptors; type 2 somatostatin receptors, which can be respectively visualized using, for example, ^{18}F-fluoro-ethyl-spiperone (FESP) and ^{68}Ga-DOTA-Tyr3-octreotide.

Great efforts have been already made in the study of the sodium iodide symporter (NIS). The ectopic expression of the NIS allows visualizing iodine accumulation inside the cell, with both PET and SPECT, since different iodine isotopes are available: iodine-124 for PET and iodine-123 for SPECT. Moreover, the induced expression of this symporter can be used as a way to deliver targeted radiotherapy with iodine-131 in tumours, thus having a new chance of treatment.

4. Clinical applications of PET molecular imaging

In clinical practice, 18-fluorine fluoro deoxyglucose (FDG) studies still represent the absolute majority. This is related to the high standardization and relative ease of synthesis of this

tracer, to the relatively long half-life of fluorine-18 which allows distribution of FDG in satellite centres, and to its well known biological behaviour.

Normal biodistribution of FDG is an image of glucose consumption in the human body. The most relevant uptake is seen in the brain which is well known to be the most sugar avid organ in the body. Since FDG is eliminated by the kidneys, a variable uptake can be seen in kidneys, urethers and the bladder. Low uptake is almost always seen in the liver and spleen. The digestive tract may show variable physiologic FDG uptake, particularly in stomach and colon. Lymphoid tissue in the oropharynx is almost always FDG avid, while heart uptake is variable and often unpredictable. Increased FDG uptake can be easily seen in skeletal muscle if the patient moves during the post-injection uptake phase. Figure 4 illustrates an attenuation corrected total-body image of a normal patient.

An index of glucose metabolism can be easily obtained using a semi-quantitative approach which is implemented in all commercially available scanners. This approach is based on the measurement of the radioactivity, expressed as Bq/ml, inside a region of interest (ROI) placed on the lesion that has to be characterized. The calculated activity is divided by the injected activity, in Bq and by the patient's body weight in grams. This value is called Standardized Uptake Value (SUV) and is reported as an absolute number. In the clinical

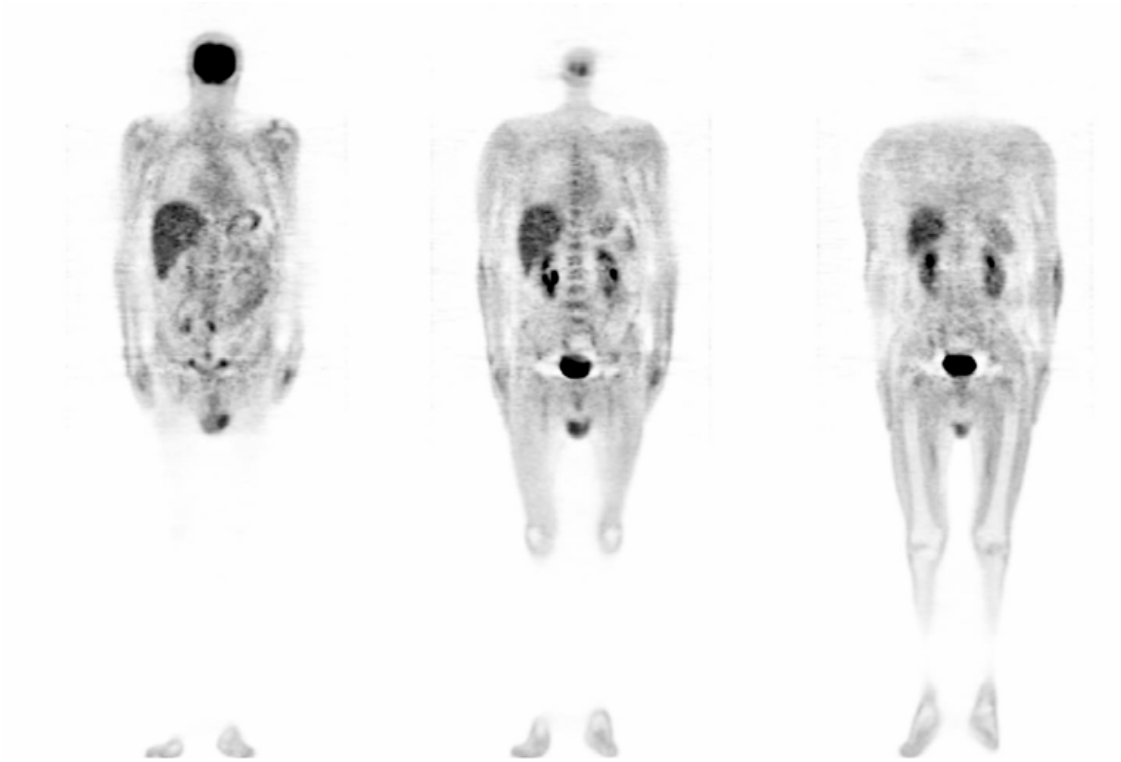

Figure 4. FDG normal distribution. Coronal reconstruction of a FDG PET total-body image. The normal bio-distribution of the radiopharmaceutical is well depicted: physiologic tracer uptake can be seen in the brain, kidneys, bladder, liver, stomach, testis.

practice the highest SUV pixel value inside a ROI (SUVmax) is commonly used to determine the degree of activity of a known lesion. The SUV value has been suggested as a prognostic factor in untreated patients affected by some neoplasms: the highest the SUV the worse the prognosis. Moreover SUV has been demonstrated to be useful in therapy assessment. The decrease in SUV value after chemotherapy or radiation therapy can be used to evaluate the effectiveness of the therapeutic regimen. Figure 5 shows the simple calculation needed to get SUV from clinical images.

4.1. Central nervous system

FDG is successfully employed in epilepsy, movement disorders and dementia evaluation. The major clinical impact has been demonstrated in Alzheimer disease, where FDG PET is able to make a diagnosis in a very early phase of the disease and to monitor response to therapy. Being a non-specific marker of neuronal damage, FDG is still a very effective tracer, revealing glucose metabolism alterations secondary to cellular damage with a high specificity and sensitivity. Clinical indications of FDG PET in the study of dementias include detection and pre-clinical diagnosis of the disease, differential diagnosis, treatment monitor and follow-up. Differential diagnosis includes other dementias or dementia-like diseases: diffuse Lewy body disease, ischemic vascular disease, front-temporal dementia, Huntington's disease, Parkinson's disease, Creuzfeldt-Jakob disease, pseudo-dementia associated with depression.

Alzheimer's disease (AD) has been identified as the commonest form of dementia in the elderly. At the present time, an estimated 4 to 5 million individuals are afflicted with the disease. The standard workup of AD relies heavily on the clinical and neuropsychological evaluation. This approach uses standardized, well-validated testing and stringent criteria. An accurate clinical diagnosis can be established in 70-90% of cases but it is more difficult to establish in the early phase of the disease, at a time when appropriate treatment regimens would be more likely have the greatest impact on disease progression and quality of life. The characteristic AD radiotracer distribution pattern for FDG PET imaging is well known. Tracer uptake is diminished to varying degrees in the posterior parietal lobes, the temporal lobes and, in some cases, the frontal lobes. The primary neocortical regions, including the sensor-motor regions, the visual cortex and the sub-cortical grey matter are relatively well preserved. FDG cerebellar metabolic uptake, in general, is diminished relative to the cortex and sub-cortical

$$SUV_{BW} = \frac{A_{c,\,tissue}}{\frac{A_o}{BW}}$$

$A_{c,tissue}$: Activity concentration in a volume (tissue) of interest enclosed in an operator generated region of interest (ROI)
A_o: Injected activity
BW: Body weight

Figure 5. Formula used to calculate Standardised Uptake Value (SUV) corrected for the patient's body weight in FDG PET images. The same formula can be used for other tracers as well

grey matter. However, in more advanced disease, the cerebellar metabolism tends to increase progressively, as part of a compensatory mechanism that involves recruitment of alternate neuronal pathways. Regional FDG distribution may be asymmetric or even unilateral in some patients. This alteration often correlates with neuropsychological testing. The overall accuracy of FDG PET imaging in detection of AD is characterized by sensitivities of 87-94% and specificities of 85-96%.

The clinical indications for performing FDG PET in the assessment of dementia are still evolving. PET imaging is a useful adjunctive technique in confirming the clinical diagnosis of AD and is even more useful in assessing patients with suspected AD. The advantage of PET is its ability to detect changes before other imaging modalities including CT, MR, and SPECT. Early detection of AD can lead to a significant reduction in the overall cost of establishing an accurate diagnosis of dementia and to the possibility for the patient to access therapies at an early stage, which might potentially delay clinical disability and improve overall quality of life.

4.2. Neoplastic diseases

FDG PET has already been accepted as an essential tool in staging, restaging and therapy response assessment of many tumours. Many of the leading cancers in the world can be evaluated with this technique: lung cancer, breast cancer, gastro-intestinal cancer, lymphoma, ovarian cancer and many other neoplasms have been demonstrated to be FDG avid. FDG PET is definitely superior to morphological imaging in terms of contrast resolution when compared to CT or magnetic resonance imaging (MRI) and the use of PET/CT combined scanners has been demonstrated to significantly increase accuracy of lesion detection, combining the high anatomical definition of CT with the high sensitivity of PET. An emerging role of PET is in the evaluation of therapy response: this technique has been demonstrated to detect very early evaluation of response to chemotherapy in many tumours. This functional approach can successfully identify responder patients, sparing useless therapy in non-responders, with a significant reduction in side effects and drugs costs. Moreover, FDG PET has been shown to be able to increase the accuracy definition of radiation therapy planning, at least in lung cancer. Again, this technique makes it possible to tailor therapies to each single patient, reducing side effects and costs.

As an example, the clinical usefulness of FDG PET for assessment of patients with Hodgkin's disease and non-Hodgkin's lymphoma (NHL) is briefly illustrated. Numerous investigations have been reported on its use for staging, restaging, and monitoring tumour response of lymphoma, with only a few exploring its value in predicting the tumour's malignancy grade. In the staging of lymphoma, PET can provide complementary information to conventional procedures such as computed tomography (CT) and bone marrow biopsy, with potential modification of stage and impact on management. It must be stressed that some data suggests the number of patients for whom PET alters therapy is small but, in the absence of modification in stages, the demonstration of a greater number of disease sites could provide important prognostic information. This would potentially be used to alter the treatment strategy in these patients.

In restaging of lymphoma, PET is able to distinguish between viable tumour and necrosis or fibrosis in the residual masses often present following treatment without any other clinical or biochemical evidence of disease. This use is probably the most important, especially in aggressive NHL and Hodgkin's lymphoma. False-positive findings at the site of residual masses may be seen, however, due to rebound thymic hyperplasia or post-therapy inflammatory changes. False-positive findings outside the site of residual masses may be

caused by infectious or inflammatory processes, including sarcoidosis. PET performed at the conclusion of treatment of patients with aggressive NHL provides a more accurate prediction of prognosis and more accurate response classifications compared with CT-based assessment. Another contribution of restaging PET is the determination of the true extent of lymphoma recurrence suspected by clinical/biochemical or radiographic evaluation.

The role of PET scanning for post-therapy surveillance without clinical, biochemical, or radiographic evidence of disease remains controversial, primarily because of the potential for false-positive findings, potentially resulting in increased cost without proven benefit from earlier PET detection of disease compared with standard surveillance methods.

Also the role of PET for monitoring response to treatment is evolving. The purpose in this setting is to provide an early and accurate assessment of response to multi-course treatment with the ultimate goal of tailoring therapy according to the information provided by the scan. Several studies demonstrated a correlation between a rapid decline of FDG uptake as early as after one or a few cycles of chemo- or chemo-immunotherapy and patient outcome. However, no published reports have yet clearly demonstrated that the PET results actually can be used to alter treatment with an improvement in outcome; early PET scanning appears to be currently performed primarily because of the prognostic information provided. The role of PET for monitoring tumour response is likely to dramatically increase once clinical trials demonstrate that the information provided by PET impacts on patient management or ultimate patient outcome.

Finally, a recently reported study in the staging and restaging of lymphoma patients must be mentioned, which showed that PET/CT was superior to PET alone. It has, therefore, been suggested that PET/CT may obviate the need for intravenous contrast used in conjunction with dedicated CT imaging of lymphoma, but this must first be confirmed in prospective clinical trials including a larger number of patients.

4.3. Infectious diseases

Increased glucose consumption is not only a characteristic of cancers, since activated cells in inflammatory and infective lesions have been demonstrated to be FDG avid. Detection of infective sites with FDG is very useful in orthopaedics applications, especially when dealing with implanted prosthesis. Similarly, detection of the source of infection in fever of unknown origin (FUO) can be successfully done with FDG PET.

It is well established that inflammatory cells have enhanced glycolysis when they are stimulated, and this has been mainly attributed to the high number of glucose transporters in these cells and partly to the enhanced affinity of these transporters for this substrate. The role of FDG-PET imaging has been extensively examined in several aseptic inflammatory processes, as well as in a wide variety of infections, and there is growing consensus about its importance in evaluating such disorders.

Assessment of suspected superimposed infection in prosthetic implants has been the subject of multiple research studies during the past several years. FDG PET has a great potential for detecting infection in hip prostheses, and to lesser extent in knee prostheses. The use of PET is advantageous over anatomic imaging modalities because it is not affected by the metal implants and it also provides better resolution images than those of the conventional nuclear medicine techniques. The criteria for diagnosing peri-prosthetic infection with FDG is essential for optimal utilization and at the present time, the potentials of this technique in the evaluation of prostheses have not been fully defined. Some authors believe that PET has excellent sensitivity but poor specificity whereas other reports indicate that the specificity of

PET is good but the sensitivity is less than optimal. These different conclusions might be the result of different interpretation criteria used by these investigators.

Because of its high sensitivity in detecting malignant lesions, infections, as well as various inflammatory processes, FDG PET has the potential to play a central role in the management of patients with FUO. However, low specificity has been demonstrated in detecting infections within the surgical wound and was attributed to the accumulation of the tracer in granulation tissue at the site of surgical intervention. Common causes of fever of unknown origin, which can be detected by PET, include a variety of malignancies and different inflammatory/infectious processes.

PET could play a major role in the management of human immunodeficiency virus infected patients, especially in those with central nervous system lesions. The high specificity of FDG PET in detecting lesions can lead to initiating an early and an appropriate treatment strategy in these severely immune-suppressed patients. It could be also effective in assessing the extent and the degree of the disease after the initial diagnosis has been made.

Based on published data PET appears to have a great potential in the diagnosis and treatment of patients with vasculitis. However, FDG-PET might not be sensitive enough to detect vasculitis of very small vessels.

Another proposed application is detecting disease activity in patients with Inflammatory Bowel Disease (IBD). However, normal FDG uptake in the bowel varies in distribution and intensity and can affect the sensitivity and specificity of the technique.

4.4. Cardiovascular disorders

Coronary artery disease (CAD) can be detected using PET tracers like ^{13}N-ammonia and rubidium-82. These approaches are able to give very accurate information in selected patients that can benefit from a very early diagnosis, like diabetics and patients with familiar diseases. Moreover, FDG-PET can be helpful in determining if an injured myocardial region would benefit from revascularization.

Despite the fact that the clinical value of cardiac PET imaging was demonstrated 20 years ago, its clinical use has been minimal, mainly due to its clinical impracticality. The increased availability of PET centres throughout the western countries changed this perspective and in the next year an increased use of PET studies is expected.

PET imaging offers a potentially high resolution of 5 to 7 mm, compared with 15 mm with SPECT, although in cardiac imaging resolution is degraded by respiratory and cardiac wall movement.

^{13}N-ammonia has been the tracer of choice for the past 2 decades. Its 10-minute half-life requires an on-site cyclotron and radiochemistry synthesis capability. In the bloodstream, ^{13}N-ammonia consists of neutral ammonia (NH3) in equilibrium with its charged ammonium ($NH4^+$) ion. The neutral NH3 molecule readily diffuses across plasma and cell membranes. Inside the cell, it re-equilibrates with its ammonium form, which is trapped in glutamine via the enzyme glutamine synthase. Another tracer is rubidium-82, produced in a commercially available generator by decay from strontium-82. This tracer is cationic and an analog of potassium. It is extracted from plasma by myocardial cells via the Na/K ATPase pump.

Published data demonstrated a mean 93% sensitivity and 92% specificity for coronary artery disease. Other studies showed higher overall sensitivity, specificity, and accuracy for PET compared with SPECT imaging. A recent comparison between ^{99m}Tc-sestamibi SPECT and

rubidium-82 PET myocardial perfusion imaging revealed a significant improvement for CAD detection accuracy with PET compared to SPECT. Despite ongoing improvements in the accuracy of SPECT, uncertainty remains in many patients even after attenuation correction, with differences in the ability of attenuation correction systems to reduce artefacts. SPECT imaging in women is challenged by the breast attenuation artefact, as well as smaller heart size. A comparison study demonstrated that in women who had undergone both stress SPECT and stress PET imaging within a period of 3 months, the SPECT and PET imaging tests had a similar high sensitivity, but the specificity for PET was significantly higher than for SPECT.

Myocardial viability is important in patients with impaired left ventricular function because of coronary artery disease with a possibility of revascularization. Combined metabolic and perfusion PET imaging, using ^{13}N-ammonia and FDG PET, offers diagnostic power in the prediction of myocardial functional recovery of viable myocardium.

4.5. Tracers beyond FDG

New tracers have been already introduced in the clinical and pre-clinical setting. These radiopharmaceuticals allow visualizing different and more specific metabolic pathways when compared to FDG. The only limitation is related to the intrinsic difficulties in the synthesis of these radiopharmaceuticals. This problem could be overcome when and if the request for these radiopharmaceuticals will increase, leading to more automated synthesis processes. Examples of these radiopharmaceuticals are: ^{11}C-choline, ^{11}C-methionine, ^{11}C-acetate, ^{18}F-fluoro-choline, ^{18}F-FLT, ^{18}F-FDOPA, ^{60}Cu-ATSM.

5. Diffusion of molecular imaging in developing countries

The main limitations of the diffusion of this technique are obviously related to the high cost of these cutting-edge technologies, to know-how diffusion and personnel education. The increasing cost of health-care in developed countries may be partly related to introduction of new technologies and this could be considered dangerous for economies of developing countries. Nonetheless, the direct introduction of these technologies in the health system of these countries could led to an immediate improvement in the management of many diseases, leading to a diffusion of a better care in the general population.

This approach could potentially lead to a reduction in the social costs of care and eventually to a general reduction of health and social costs.

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Nuclear medicine procedures in the postgenomic era

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Abstract. Assessment of gene function following the completion of human genome sequencing may be done using radionuclide imaging procedures. These procedures are needed for the evaluation of genetically manipulated animals or newly designed biomolecules which requires a thorough understanding of physiology, biochemistry and pharmacology. The experimental approaches will involve many new technologies including *in vivo* imaging with SPECT and PET. Nuclear medicine procedures may be applied for the determination of gene function and regulation using established and new tracers or using *in vivo* reporter genes such as genes encoding enzymes, receptors, antigens or transporters. Visualization of *in vivo* reporter gene expression can be done using radiolabelled substrates, antibodies or ligands. Combinations of specific promoters and in vivo reporter genes may deliver information about the regulation of the corresponding genes. Furthermore, protein-protein interactions and activation of signal transduction pathways may be visualized non-invasively. The role of radiolabelled antisense molecules for the analysis of mRNA content has to be investigated. However, possible applications are therapeutic intervention using triplex oligonucleotides with therapeutic isotopes which can be brought near to specific DNA sequences to induce DNA strand breaks at selected loci. Imaging of labelled siRNA's makes sense if these are used for therapeutic purposes in order to assess the delivery of these new drugs to their target tissue. In gene therapy based on the transfer and expression of suicide genes, usually gene coding for the non-mammalian enzymes, the Herpes Simplex virus thymidine kinase (HSVtk) or the yeast and bacterial cytosine deaminase (CD), have been used. After infection of the tumour with the recombinant virus, a non-toxic prodrug is applied systemically, which is subsequently converted to a toxic metabolite by the recombinant gene product. Employing a radiolabelled prodrug and scintigraphic procedures it is possible to determine the functional activity of the recombinant enzyme *in vivo*. This information can be used to establish a therapeutic window of maximal gene expression and consecutive drug administration. If the gene therapy approach is based on the transduction of receptor genes, the recombinant gene expression in tumour cells is monitored using radiolabelled ligands. Transfer of transporter genes such as the sodium iodide transporter may also permit the visualization of transduction via accumulation of iodide or pertechnetate in the targeted tissue. Pharmacogenomics will identify new surrogate markers for therapy monitoring which may represent potential new tracers for imaging. Also drug distribution studies for new therapeutic biomolecules are needed at least during preclinical stages of drug development. New treatment modalities such as gene therapy with suicide genes will need procedures for therapy planning and monitoring. Finally, new biomolecules will be developed by bioengineering methods which may be used for isotope-based diagnosis and treatment of disease.

1. Introduction

After the sequencing of the human genome has been completed, a tremendous amount of new research is needed to make sense of the sequence information which is now available. The basic challenges are: finding the genes, locating their coding regions and predicting their functions. The next steps following gene identification are designed to figure out the properties that specific genes encode and what they do for a living organism. Gene mapping and sequencing delivers information about linkages, genome organization, protein complement, gene regulation, phylogeny and evolution. One of the main problems is to package the huge amount of sequences and expression profiles generated by new array methods into useful biological knowledge [1]. This may result in new diagnostic and therapeutic procedures which include visualization of and interference with gene transcription and the development of new biomolecules useful for diagnosis and treatment.

2. Analysis of gene expression from chips to images

The estimation of gene function using the tools of the genome program has been referred to as "functional genomics" which can be seen as describing the processes leading from a gene's physical structure and its regulation to the gene's role in the whole organism. Many studies in functional genomics are performed by analysis of differential gene expression using methods such as DNA chip technology. These methods are used to evaluate changes in the transcription of many or all genes of an organism at the same time in order to investigate genetic pathways for normal development and disease.

A possible tool for the non-invasive detection of gene expression may be antisense imaging. Antisense RNA and DNA techniques have been originally developed to modulate the gene expression in a specific manner. These techniques originated from early studies in bacteria demonstrating that these organisms are able to regulate their gene replication and expression by the production of small complementary RNA molecules in an antisense (opposite) direction. Base pairing between the oligonucleotide and the corresponding target mRNA leads to highly specific binding and specific interaction with protein synthesis. Thereafter, several laboratories showed that synthetic oligonucleotides complementary to mRNA sequences could downregulate the translation of various oncogenes in cells [2-3]. However, besides their use as potential therapeutics for specific interaction with RNA processing radiolabelled oligonucleotides, have been proposed for diagnostic imaging and the therapy of tumours. Assuming a total human gene number between 30 000 and 35 000 calculations which take into account alternative polyadenylation and alternative splicing result in a mRNA number between 46 000 and 85 000 [4] which can theoretically be used for diagnostic or therapeutic purposes. It is expected that an oligonucleotide with more than 12 (12-mer) nucleobases represents a unique sequence in the whole genome [5]. Since these short oligonucleotides can easily be produced antisense imaging using radiolabelled oligonucleotides may in principle offer a huge amount of new specific tracers. Prerequisites for the use of radiolabelled antisense oligonucleotides are ease of synthesis, stability in vivo, up-take into the cell, accumulation of the oligonucleotide inside the cell, interaction with the target structure, and minimal non-specific interaction with other macromolecules. For the stability of radiolabelled antisense molecules, nuclease resistance of the oligonucleotide, stability of the oligolinker complex and a stable binding of the radionuclide to the complex are required. In this respect, modifications of the phosphodiester backbone such as phosphorothioates, methylphosphonates, peptide nucleic acids or gapmers (mixed backbone oligonucleotides) result in at least a partial loss in cleavage by enzymes such as RNAse H.

Evidence has been presented of a receptor coupled endocytosis of low capacity as the mechanism by which oligonucleotides enter cells [6-7]. Sub-cellular fractionation experiments showed a sequestration of the oligonucleotides in the nuclei and the mitochondria of HeLa cells [7]. This fractionation, the problems with the in vivo stability of the oligonucleotides, as well as the stability of the hybrid oligo-RNA structures, are severe obstacles to successful imaging of gene expression. Furthermore, binding to other polyanions such as heparin based on charge interaction may cause unspecific signals.

However, accumulation of ^{111}In-labelled c-myc antisense probes with a phosphorothioate backbone was reported in mice bearing c-myc overexpressing mammary tumours [8]. Tumour imaging was also possible with a transforming growth factor α antisense oligonucleotide or antisense phosphorothioate oligodeoxynucleotide for the mRNA of glial fibrillary acidic protein [9-11]. Also in rat glioma cells permanently transfected with the luciferase gene, autoradiography showed accumulation of a ^{125}I-labelled antisense peptide nucleic acid

targeted to the initiation codon of the luciferase mRNA [12]. Furthermore, positron emission tomography (PET) was used for the assessment of the biodistribution and kinetics of ^{18}F-labelled oligonucleotides [13]. In addition, ^{90}Y labelled phosphorothioate antisense oligonucleotides may be applied as targeted radionuclide therapeutic agents for malignant tumours as was done for a phosphorothioate antisense oligonucleotide complementary to the translation start region of the N-myc oncogene mRNA [14]. The resulting ^{90}Y antisense oligonucleotide hybridized specifically to a complementary phosphorodiester sense oligonucleotide.

However, current data show that the transcriptome obtained with messenger RNA (mRNA) profiling for the characterization of cellular phenotypes does not faithfully represent the proteome because the mRNA content seems to be a poor indicator of the corresponding protein levels [15-17]. Direct comparison of mRNA and protein levels in mammalian cells either for several genes in one tissue or for one gene product in many cell types reveals only poor correlations (0.5 or lower) with up to 30-fold variations. This might lead to misinterpretation of mRNA profiling results.

Furthermore, a substantial fraction of interesting intracellular events is located at the protein level, for example, operating primarily through phosphorylation/ dephosphorylation and the migration of proteins. Also proteolytic modifications of membrane-bound precursors appear to regulate the release of a large series of extracellular signals such as angiotensin, tumour necrosis factor, and others. mRNA is much more labile than DNA leading to spontaneous chemical degradation and to degradation by enzymes. Degradation may be dependent on the specific sequence, resulting in non-uniform degradation of RNA, which introduces quantitative biases that are dependent on the time after the onset of tissue stress or death. In contrast, proteins are generally more stable, and exhibit slower turnover rates in most tissues.

Expression profiling approaches data would be more useful, if mRNA samples could be enriched for transcripts that are being translated [18]. This can be achieved by fractionation of cytoplasmic extracts in sucrose gradients which leads to the separation of free mRNPs (ribonucleoprotein particles) from mRNAs in ribosomal preinitiation complexes and from mRNAs loaded with ribosomes (polysomes). Since only the polysomes represent actively translated transcripts, this fraction should be directly correlated with de novo synthesized proteins. This method assumes that translational control predominantly occurs at the initiation step. Thus polysome-bound mRNA profiling should provide a closer representation of the proteome than does profiling of total mRNA. Although this hypothesis is supported by measurements of total protein synthesis rates and overall polysome-bound mRNA levels [19], it remains to be proven on a proteome-wide scale. Furthermore, polysome-bound mRNA profiling cannot be used to study changes affecting protein levels by proteolysis, post-translational modifications, subcellular localization or protein degradation. This type of analysis is restricted to proteomics.

Since protein levels often do not reflect mRNA levels, antisense imaging may be not a generally applicable approach. Polysome imaging with nuclear medicine procedures has not been tried to date or even may be not possible. Therefore, antisense imaging for the determination of transcription by hybridization of the labelled antisense probe to the target mRNA makes sense in cases where a clear correlation of mRNA and protein exists. Correspondingly successful imaging was possible in cases where the expression of the protein was proven [8-12]. If no correlation between mRNA and protein exists, the diagnostic use of antisense imaging is questionable. It may be expected that rather therapeutic applications using triplex oligonucleotides with therapeutic isotopes would make sense. In that case Auger

electron emitters can be brought near to specific DNA sequences to induce DNA strand breaks at selected loci.

3. Functional studies in animals bearing mutations

Usually, producing a knock-out mutation or altering the expression of a gene gives rise to a phenotype that provides insights into the function of a gene. Besides these genotype-driven mutations there is increasing need for phenotype-driven mutations (for example using the alkylating agent N-ethyl-N-nitrosurea) to identify genes that are involved in specific kinds of disease. This approach needs no assumptions with respect to which genes and what kinds of mutations are involved in a particular phenotype or disease. In order to maximize the efficiency of the related experiments it will be necessary to develop multiple assays working at different levels of description (morphologic, physiologic, biochemical or behavioural) to detect a large number of different phenotypes in a given set of mutagenized mice. The screening of a mutagenized population is usually done with phenotypically visible coat colour markers in combination with selection genes as neomycin resistance gene and the Herpes Simplex virus thymidine kinase (HSVtk) gene. However, using genes as HSVtk or others in combination with scintigraphic imaging as non-invasive in vivo reporters may be an attracting alternative.

4. Use of in vivo reporter genes

Commonly used reporter genes as β-galactosidase, chloramphenicol-acetyltransferase, green fluorescent protein or luciferase play critical roles in investigating the mechanisms of gene expression in transgenic animals and in developing gene delivery systems for gene therapy. However, measuring expression of these reporter genes often requires biopsy or death or cannot be used for the non-invasive imaging of deeper structures in the body. In vivo reporter genes can be visualized non-invasively using radiolabelled molecules. In this respect, genes encoding for enzymes, receptors, antigens and transporters have been used. Enzyme activity can be assessed by the accumulation of the metabolites of radiolabelled specific substrates, receptors by the binding and /or internalization of ligands, antigens by binding of antibodies and transporters by the accumulation of their substrates. Since expression of the HSVtk gene leads to phosphorylation of specific substrates and to the accumulation of the resulting negatively charged metabolite, this gene can be used as an in vivo reporter gene [20-26]. Using radiolabelled specific HSVtk substrates, a significantly higher uptake was found in HSVtk-expressing cells as compared to the wild type controls. There was a significant positive correlation between the percent injected dose of [^{131}I] 5-iodo-2'-fluoro-2'-deoxy-1-b-D-arabinofuranosyluracil (FIAU) and [^{18}F] fluoroganciclovir retained per gram of tissue and the levels of HSVtk expression. The amount of tracer uptake in the tumours was correlated to the in vitro ganciclovir sensitivity of the cell lines which were transplanted in these animals. A general problem is the fact that the affinity of these specific substrates for the nucleoside transport systems as well as for the enzyme is rather low which may be a limiting factor for cellular accumulation. Therefore, at present the ideal tracer for HSVtk imaging has not been identified and more efforts have to be done to synthesize radiolabelled compounds with improved biochemical properties.

In order to improve the detection of low levels of PET reporter gene expression, a mutant Herpes Simplex virus type 1 thymidine kinase (HSV1-sr39tk) has been used as an in vivo reporter gene for positron emission tomography [27]. Successful transfer of this mutant gene resulted in enhanced uptake of the specific substrates [8-^{3}H] penciclovir, and

8-[^{18}F]fluoropenciclovir in C6 rat glioma cells with a twofold increase in accumulation compared with wild type HSVtk-expressing tumour cells.

However, other genes may also be candidates for the in vivo detection of gene transfer. The dopamine D2 receptor gene represents an endogenous gene which is not likely to invoke an immune response. Furthermore, the corresponding tracer 3-(2'-[^{18}F]-fluoroethyl) spiperone (FESP) rapidly crosses the blood-brain-barrier, can be produced at high specific activity and is currently used in patients. The tracer uptake in nude mice after transfection with an adenoviral-directed hepatic gene delivery system and also in transplanted stable tumour cells was proportional to in vitro data of hepatic FESP accumulation, dopamine receptor ligand binding and the D2 receptor mRNA [28]. Also tumours modified to express the D2 receptor retained significantly more FESP than wild type tumours. In modified non-small cell lung, cell lines expressing the human type 2 somatostatin receptor and transplanted in nude mice images were obtained using an somatostatin-avid peptide (P829), that was radiolabelled to high specific activity with ^{99}mTc or ^{188}Re [29]. In the genetically modified tumours a 5- to 10-fold greater accumulation of both radiolabelled P829 peptides as compared to the control tumours was observed. The ^{188}Re-labelled peptide revealed similar results and has the additional advantage of energetic β decay with a potential use for therapy.

To overcome the limitation of low expression of tumour-associated antigens on target cells for radioimmunotherapy, the gene for the human carcinoembryonic antigen (CEA) was transferred in a human glioma cell line resulting in high levels of CEA expression [30]. In these modified tumour cells high binding of a ^{131}I-labelled CEA antibody was observed in vitro as well as by scintigraphic imaging.

Another approach is based on the in vivo transchelation of oxotechnetate to a polypeptide-motif from a biocompatible complex with a higher dissociation constant than that of a dicglycilcysteine complex. It has been shown that synthetic peptides and recombinant proteins like a modified green fluorescence protein (GFP) can bind oxotechnetate with high efficiency [31]. In these experiments rats were injected i.m. with synthetic peptides bearing a diglycylcysteine (GGC) binding motif. One hour later ^{99m}Tc-glucoheptonate was injected i.v. and the accumulation was measured by scintigraphy. The peptides with three metal-binding GGC motifs showed a threefold higher accumulation as compared to the controls. This principle can also be applied to recombinant proteins which appear at the plasma membrane [32].

Transfer of the human sodium iodide symporter (hNIS) gene into Moris hepatoma cells caused a significant increase in iodide uptake (by a factor of 84 to 235) with a peak after 1 hour incubation. The radioactive iodide was concentrated in the cells to values up to 105-fold more than in the medium [33]. This is far more than in normal thyroid tissue where an I-concentration gradient of 30-fold in FRTL5 cells and 20- to 40-fold in the thyroid gland in vivo has been described. However, a rapid efflux (80%) was observed in hNIS-expressing hepatoma cells during the first 10 minutes indicating that no organification of the radioactive iodide occurred. Animal studies with wild type and hNIS-expressing tumours in rats showed similar results with a maximum uptake after 1 hour and a continuous disappearance of the radioactivity out of the body as well as of the hNIS-expressing tumours [33]. Although the NIS activity is asymmetrical favouring iodide influx, there is obviously an efflux activity with the consequence that in cells that do not organify iodide, the concentration of intracellular iodide will drop proportionally to the external iodide concentration. After administration of 0.4 mCi ^{131}I this resulted in an absorbed dose of 35 mGy (wild type tumour) and 592 mGy (hNIS-expressing tumour). Therefore, the use of the hNIS gene alone for radioiodine therapy

of non-thyroid tumours seems questionable, but the hNIS gene may be used together with ^{121}I, ^{124}I or even with ^{99m}Tc-pertechnetate as a simple reporter system for the visualization of other genes in bicistronic vectors which allows co-expression of two different genes [34].

Of all these reporter genes described, the sodium iodide symporter gene may present the advantage that it is not likely to interact with underlying cell biochemistry. Iodide is not metabolized in most tissues and, although sodium influx may be a concern, no effects have been observed to date [35]. The HSVtk gene may alter the cellular behaviour towards apoptosis by changes in the deoxyribonucleotide triphosphate (dNTP) pool [36], while antigens may cause immunoreactivity and receptors may result in second messenger activation such as triggering signal transduction pathways. However, these possible interactions have to be studied in detail in future experiments. For the dopamine 2 receptor system a mutant gene has been applied which shows uncoupling of signal transduction [37].

5. Analysis of gene regulation by reporter assays

Functional genomics partly relies on a comparison of sequences near coding regions in diverged organisms assuming that nucleotides conserved in noncoding regions between these pairs of organisms identify functional sites which typically are response elements for regulatory proteins. Among genes which exhibit correlated expression patterns across a large variety of biological conditions, a significant fraction is expected to be co-regulated i.e. responsive to common expression factors. However, the prediction of promoter locations and properties as well as analyses for the precise identification of intron/exon architecture and boundaries of gene transcripts still face unacceptable uncertainties [38-39].

With the increasing availability of intrinsically fluorescent proteins that can be genetically fused to virtually any protein of interest, their application as fluorescent biosensors has extended to dynamic imaging studies of cellular biochemistry even at the level of organelles or compartments participating in specific processes [40]. On the supracellular level, fluorescence imaging allows the determination of cell-to-cell variation, the extent of variation in cellular responses and the mapping of processes in multicellular tissues. Furthermore, visualization of intracellular gradients in enzymatic activities, such as phosphorylation and GTPase activity, can now be related to morphogenetic processes, where the distribution of activity shapes the cellular response.

However, for the examination of whole organisms and especially of organ systems in deeper parts of the body in vivo reporter systems are promising. Biological systems are more complex than cell cultures because external stimuli may affect and trigger cells. Therefore, noninvasive dynamic in vivo measurements are needed to study gene regulation in the physiological context of complex organisms. These in vivo reporters may be used also for the characterization of promoter regulation involved in signal transduction, gene regulation during changes of the physiological environment and gene regulation during pharmacological intervention. This may be done by combining specific promoter elements with an in vivo reporter gene. Furthermore, the functional characterization of new genes will result in new diagnostic targets and possibly also in new tracers for their visualization that may be substrates for enzymes or transporters, ligands for receptors or antibodies for antigens. However, there may be concerns about the image resolution even when animal scanners are used. Therefore, autoradiography or fluorescence resonance energy transfer (FRET) represent alternatives in cases where resolution at the μm range is required.

6. Design of new biomolecules for diagnosis and therapy

Through the accumulation of genomics and proteomics data, novel biomolecules may be discovered or designed on a rational basis in diverse areas including pharmaceutical, agricultural, industrial and environmental applications. This can be achieved through directed genome evolution, metabolic pathway engineering, protein engineering, analyses of functional genomics and proteomics, high throughput screening techniques and the development of bio-process technology [41]. The products of this process will be monoclonal antibodies, vaccines, enzymes, antibiotics, therapeutic peptides and others. Besides bioinformatics, functional genomics, proteomics, protein chemistry and engineering, the methods involved include also recombinant techniques as random mutation DNA shuffling and phage-displays, metabolic pathway engineering including metabolic flux analysis and bioprocess engineering which develops technologies needed for the production of the desired high-value biomolecules.

Two techniques are important for the design of new biomolecules: DNA shuffling and phage-display libraries. DNA shuffling mimics natural recombination by allowing in vitro homologous recombination of DNA [42-43]. Therefore, a population of related genes is randomly fragmented and subjected to denaturation and hybridization, followed by the extension of 5' overhang fragments by Taq DNA polymerase. A DNA recombination occurs when a fragment derived from one template primes a template with different sequences. The applications of this method include improvement of enzyme properties, development of altered metabolism pathways, antibiotics and pharmaceutical proteins, development of plasmids or viruses for novel vaccines and gene therapy applications. Genes from multiple parents and even from different species can be shuffled in a single step in operations that do not occur in nature but may be very useful for the development of diagnostic and therapeutic approaches.

The principle of phage-displayed peptide libraries is the display of the peptide libraries fused with the carboxy-terminal domain of the minor coat protein, gene III protein fragment, on the surface of a filamentous phage. The relevant molecule is then directly detected and screened using the target molecules and amplified after infection of E. coli. This allows a rapid selection (within weeks) of particular clones from large pools (> 1010 clones), and determination of the amino acid sequence of a peptide displayed on a phage by sequencing the relevant section of the phage genome. This technique has been employed for searching antibodies, receptors for new drug discovery and cancer therapy, either as an antagonist or an agonist of a natural ligand-receptor interaction [44-45] and custom-made enzymes for gene therapy.

One of the main goals of biocatalyst engineering is to endow them with new features that are not found in natural sequences because they confer no evolutionary advantage [46]. The design of a biocatalyst involves two main steps which can be iterative: making a set of mutant biocatalysts and searching that set of mutants with the desired properties. In this stage of development isotope based methods are needed to assess binding characteristics of antibodies or ligands or measure new transport or enzyme functions in vivo. Furthermore, in later stages the coupling of antibodies or peptides with α or β emitters may be used for therapeutic purposes.

7. Therapy monitoring

For therapy monitoring, proteomics may deliver new means for the evaluation of therapeutic effects on the target tissue as well as on other tissues. This is based on the assumption that many therapeutic drugs act through mechanisms involving perturbations of protein expression and, therefore, a successful drug could be defined as one that restores the expression levels of a cell or an organ to the normal state [47]. Using this approach drugs can be compared with respect to their effectiveness in restoring the normal protein expression. These novels "surrogate markers" for functional measurements will provide quantitative data of the drug's influence on the disease process, whereas standard clinical trials depend primarily on improvements in clinical signs. This relies on strong functional relation-ships between drug treatment, protein expression and resulting physiological effects where the real therapeutic mechanism often consists of modulations in protein gene expression occurring as a secondary result. Therefore, it is expected that drugs acting through similar mechanisms ought to produce similar gene expression effects and, conversely, different mechanisms should produce distinct expression effects.

Also measurement of the pattern of protein changes can be used to describe the mechanism of action. Proteomic offers the opportunity to obtain complimentary information to genomic-based technologies for the identification and validation of protein targets in following time-dependent changes in protein expression levels which result from selective interaction with specific biological pathways and identifying protein networks (functional proteomics). Changes in protein expression or function could also serve as targets for noninvasive imaging procedures. Although nuclear medicine procedures are not large scale measurements by defining leading changes in protein content or function, it may be possible to use radiolabelled ligands or substrates to assess the drug's effects on specific parts of the proteome. This may be done using established tracers for new therapies or using new tracers which have been identified either by functional studies of new genes or by analysis of changes in expression or functional patterns by the high-throughput methods of functional genomics.

As an example, measurements of tumour perfusion and tumour metabolism have been performed to assess the efficacy of suicide gene therapy. Tumour perfusion, as measured in ganciclovir (GCV) treated HSVtk-expressing KBALB tumours after intravenous administration of [^{99m}Tc]HMPAO, increased by 206% at day 2 after the onset of ganciclovir treatment [48]. In the same animals, the accumulation of the hypoxia tracer [^{3}H]misonidazole decreased to 34% at day 3, indicating that the tumour tissue had become less hypoxic during ganciclovir treatment.

The ^{18}F-fluorodeoxyglucose (FDG) uptake has demonstrated to be a useful and very sensitive parameter for the evaluation of glucose metabolism during or shortly after treatment of malignant tumours. Dynamic PET measurements of 18FDG uptake in rats bearing HSVtk-expressing hepatomas revealed an uncoupling of FDG transport and phosphorylation with enhanced transport values and a normal phorphorylation rate after two days of GCV treatment [49]. These tumours showed a significant increase of the glucose transporter 1 (GLUT1) as demonstrated by immunohistochemistry [50]. The increase in FDG transport normalized after four days whereas the phosphorylation rate increased. As an underlying mechanism, a redistribution of the glucose transport protein from intracellular pools to the plasma membrane may be considered and is observed in cell culture studies as a general reaction to cellular stress. Consequently, inhibition of glucose transport by cytochalasin B or competition with deoxyglucose increased apoptosis [50].

Besides these established monitoring procedures, we may expect new biochemical pathways emerging from proteomics research leading to the use of radiolabelled substrates for enzymes, transport systems or specific structures on cell membranes.

8. Conclusion

A huge amount of new molecular structures have been cloned and are now available as potential novel diagnostic or drug discovery targets. Therefore, the target selection and validation has become the most critical component in this process. Ultimately, the disciplines of functional genomics and proteomics have their foundation in the physiological, biochemical and pharmacological sciences. The evaluation of genetically manipulated animals or newly designed biomolecules will require a thorough understanding of physiology, biochemistry and pharmacology and the experimental approaches will involve many new technologies including in vivo imaging with MRI and PET [51]. Nuclear medicine procedures may be applied for the determination of gene function and regulation using established or new tracers, for example in knockout mice or in transgenic animals. The measurement of gene regulation may also be done using in vivo reporter genes such as enzymes, receptors, antigens or transporters. Intracellular signalling has been visualized in vitro with combinations of specific regulatory elements (promoters, enhancers) and reporter genes such as the secreted alkaline phosphatase (SEAP) downstream of several copies of specific transcription factor binding sequences [52]. This approach may be extended for in vivo detection using in vivo reporters instead of SEAP. Pharmacogenomics will identify new surrogate markers for therapy monitoring which may represent potential new tracers for imaging. Also drug distribution studies for new biomolecules are needed to fasten drug approval at least in preclinical stages of drug development. Finally, bioengineering will lead to the design of new bio-molecules by methods such as DNA shuffling or phage-display procedures which may be used for new approaches in isotope-based diagnosis and treatment of disease.

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Use of molecular nuclear methods in communicable diseases: From nucleic acid hybridization to proteomics

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Abstract. In spite of decades of intensive research on communicable diseases, infection agents still remain a major cause of morbidity and mortality in humans. These rates have driven a major resurgence in biological research efforts for the identification of new targets for vaccine development, drugs and diagnostics assays products. Molecular nuclear techniques focused in nucleic acid hybridization and DNA amplification and more recently proteomic approaches are employed to address questions related to communicable diseases. In nucleic acid hybridization, denatured DNA or RNA is immobilized on an inert support, so that bound sequences are available for hybridization with an added nucleic acid radioactive probe to facilitate analysis. After extensive washing, hybrids are detected by autoradiography or phosphoimager analyzer. Polymerase chain reaction (PCR), is a very sensitive method for nucleic acid amplification used when the targets are scarce in the cells or in clinical material. Differently, the challenge of proteome analysis lies with the task of achieving a combination of high-throughput screening while maintaining high sensitivity for the detection of low copy number proteins. Many different detection technologies have been developed to improve visualization of proteins in protein analysis and radiolabelling is one of the strategies for protein detection. For metabolic experiments, proteins must be labelled with an appropriate radioactive isotope in vivo prior to isolation by electrophoretic analysis. Additionally, protein phosphorylation, an important post-translational modification, is analyzed by using 32P for in vivo labelling of phosphorylated proteins and further identification by mass spectrometry. The major problems which limit proteomic studies deal with the comparative analysis of 2DE gel images. In conventional methodology, the protein samples to be compared are separated independently on different gels. Using a 2DE image analysis software spots are matched between gels for comparison, but it is a difficult task as protein patterns are never perfectly super imposable. To circumvent this problem a modified 2DE technique called differential gel exposure (DifExpo), an alternative method to DIGE, was developed. In DifExpo the sample are differentiated from each other by in vivo radiolabelling, using14C and 3H - isotopes. After 2DE separation and transfer to a polyvinylidene fluor (PVDF) membrane, the ratio 3H/14C of each protein spot is determined by exposure to two types of image plates, one sensitive to 14C and another to both 14C and 3H. Accelerator mass spectrometry (AMS) and multi-photon detection (MDP) are also methods used to improve greatly the sensitivity of detection and quantification of radiolabels in 2DE gels analysis.

1. Introduction

For several years molecular biology and genomics have been disconnected from the Millennium Development Goals (MDGs). More recently, an alignment of these sciences with the United Nations development agenda has been sought. A framework for collective action worldwide has been proposed by several UN agencies using tools based on molecular methods, and this is an important benchmark to illustrate the potential of science to close the gap between northern and southern countries and to show how emerging technology can be incorporated in a variety of approaches used to meet the MDGs.

Among these objectives, one can find the reduction of extreme poverty and hunger, the achievement of universal primary education and gender equity, the reduction of child and maternal mortality, the halting of the spread of Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS), malaria and other diseases, and the access to safe drinking water. All the aforementioned goals should be involved in an environment of sustainability, and for that a global partnership for development should be involved.

Obviously, molecular biology cannot address all these aspects. However, there are some settings where a molecular approach can play an important role. Molecular diagnosis, recombinant vaccines, drug delivery, definition of pathogen genomes, protection against sexually transmitted diseases, bioinformatics, nutritionally enriched genetically modified crops, recombinant therapeutic proteins, combinatorial chemistry and bioremediation were identified as the major helpful tools.

Regarding communicable diseases, the scary scenario is that, for instance, 55% of the HIV+ individuals in sub-Saharan Africa are women; HIV prevalence is 5 times higher in teenage girls than in boys; 11 million children die before meeting their fifth birthday; over 500,000 maternal deaths occur per year, most of them due to infections; and HIV/AIDS, malaria and tuberculosis are responsible for 40% of all deaths in the developing world, meaning 5 million people per year. This same rate of deaths per year is attributed to waterborne diseases.

The World Health Organization has defined a list of communicable disease that should be targeted. Among these are African trypanosomiasis, Chagas disease, dengue, leishmaniasis, leprosy, lymphatic filariasis, malaria, onchocerciasis, schistosomiasis, tuberculosis and AIDS.

The needs are the identification of new targets from the microorganisms (i) involved in pathogenesis and risk factors, (ii) for drugs, (iii) vaccines development and (iv) diagnostic assays production. Drug resistance is another problem that has been increasing during the last few years in several of these diseases and population genetics may help to better understand the diversity of these pathogens affecting humans in the developing world.

It seems that there is an optimization when communicable disease targets are addressed by molecular nuclear techniques varying from hybridization using molecular probes to proteomics. In this scenario, both isotopic and non-isotopic labelling of nucleic acid molecules plays an important role. For labelling proteins, radionuclides are preferentially used. In this chapter, a brief description of the use of these techniques is given, focusing the attention upon isotopic labelling.

Traditionally molecular nuclear methods to address problems related to communicable diseases focused on DNA amplification and molecular hybridization using radiolabelled probes. The protein chemistry, as a part of the "forward genetics"[1] approaches and together with molecular biology methods, allows it to make the link between the observed activity or function of a biochemically isolated protein and the gene that encoded it. The search to develop more sensitive and reliable methods to sequence and identify proteins was, therefore, the principal objective. With the advent of the first results from large-scale sequencing projects [1], the development of "reverse genetics"[2] approaches was established. Such approaches included the study of coordinated gene expression patterns in different cellular states [2] and further clustering of mRNA species [3]. These days, the technical edges are broader, overlapping and complementing genomics and proteomics fields. In this report, the use of the aforementioned techniques is described and some applications in infectious diseases are depicted.

[1] Approaches attempted to move from an observed phenotype to the genes and their products that caused that phenotype.
[2] Approaches attempted to move from the gene sequence to function and phenotype.

2. Molecular nuclear techniques

Nucleic acid hybridization, the formation of a duplex between two complementary nucleotide sequences, is the basis for a range of techniques now in widespread use in modern biology. In this sense, hybridization is an essential methodology for molecular biology studies. The applications of the hybridization technique range from fundamental research in molecular biology to clinical diagnosis of human infectious. The process which underlies all of the methods based on molecular hybridization relies on two basic paradigms: two sequences involved in duplex formation must have a degree of complementary, and the stability of the duplex formed depends on the extent of the complementary. Nowadays, most hybridization experiments are performed with both radioactive or non-radioactive probes and filter-bound nucleic acids.

Denatured DNA or RNA is immobilized on an inert support, for example nylon membranes, in such a way that self-annealing is prevented, yet bound sequences are available for hybridization with an added nucleic acid probe. To facilitate analysis, the probe is labelled, usually with ^{32}P. Hybridization is followed by extensive washing of the filter to remove the unreacted probe. Detection of hybrids is usually by autoradiography or a phosphoimager. The procedure is widely applicable, being used for Southern blot and dot blot hybridization for instance.

Sometimes, the targets that are being sought are scarce in the cells or in the clinical samples, such as a small number of parasites in the available clinical material. To circumvent this scenario, the polymerase chain reaction (PCR), an *in vitro* method of nucleic acid amplification, was a landmark regarding communicable diseases diagnosis. It involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. These primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase enzyme proceeds across the region between the primers. Since the extension products themselves are also complementary to and capable of binding primers, successive cycles of amplification essentially double the amount of the target DNA synthesized in the previous cycle. The result is an exponential accumulation of the specific target fragment, approximately 2^n, where n is the number of cycles of amplification performed. Polymerase chain reaction (PCR) is so sensitive that a single DNA molecule can be amplified, and single-copy genes are routinely extracted out of complex mixtures of genomic sequences and visualized as distinct bands on agarose gels.

The analytical specificity of the molecular methods is dependent on how the probes and primers anneal to the target site. Besides probe sequence, combination of two other parameters during hybridization guarantees annealing specificity: temperature and salt concentration. Accurate balance of these factors ensures that probes anneal to the specific region of nucleic acid and, consequently, increases specificity. Usually probes used in the diagnosis of infectious diseases are applied to the detection of microorganisms and present no cross-hybridization with human DNA, therefore being suitable for clinical application. On the other hand, the sensitivity of the PCR is guaranteed due to the exponential amplification of a determined target. The joining of the sensitivity of the PCR plus the specificity of molecular hybridization of probes makes the coupling of these two approaches ideal tools for the diagnosis of infectious diseases.

Combined, these aspects allow ready detection of single pathogenic organisms, an accomplishment provided before by *in vitro* culturing of such pathogens. However, since the exponential amplification of PCR is catalyzed by a biochemically simple cyclical process

requiring less than minutes per cycle, this procedure promises to supplant the culturing of pathogen that frequently requires a total of days to weeks to reach a final conclusion. Furthermore, pathogens not capable of *in vitro* propagation, such as human papilomavirus, are refractory to detection using the culturing approach. Antibodies used in the direct search of a given pathogen typically recognize antigens found in multiple copies on the microorganism to circumvent the need to replicate the agent. Unfortunately, the cross-reactivity of these antibodies with host antigens and other pathogens has compromised the convenient and broader use of these diagnostic reagents. In addition, some viruses establish a latent infection in which active viral replication is substantially attenuated thereby making difficult the protein detection process.

Although most diagnostic assays are based on sample collection by non-invasive means, invasive sampling is sometimes unavoidable. By definition, optimal invasive sampling requires the collection of minuscule quantities of material. Even needle punch biopsies and aspirates of various types provide sufficient material for molecular analysis.

3. Proteomics analysis

Proteomics is a particularly rich source of biological information because proteins are involved in almost all biological activities and contribute greatly to the understanding of biological systems. In this context, the initial goal of proteomics was the rapid identification of all proteins expressed by a cell or tissue. Proteomic research is directed toward the systematic determination of diverse properties of proteins: sequence, quantity, modifications, interactions, activities, distribution and structure. Like other technologies that pursue the investigation of a biological system or process, proteomics is the consequence of the results obtained from mapping and sequencing the complete genome of many species.

The development of more sensitive and reliable methods to sequence and identify proteins has been sought for the last three decades. With the advent of the first results from large-scale sequencing projects, studies of coordinated gene expression patterns in different cellular states could be approached. Long before global gene expression analysis was possible, protein science already attempted to develop global approaches to the quantitative analysis of protein expression patterns throughout high-resolution two-dimensional gel electrophoresis (2DE, Figure 1). This technique intends to generate reproducible protein arrays displaying large numbers of separated proteins and indicating their quantities and in fact, it is proposed to analyze information in a way similar to current strategies for analyzing microarrays data.

With the rapid advances in mass spectrometry (MS) technologies, sequence databases and data base search tools as well as with the improvement of 2DE technologies itself, proteomics became a reality. Currently, 2DE combined with protein identification by MS is widely used in proteomics (Figure 2).

Among all applications of proteomics, the identification of differential protein expression between two samples of microorganisms, conditions or treatments can provide unique markers of biological activity. These are still some limiting points that need improvement, such as the ideal sensitivity to deal with low abundance proteins and limited sample amounts, greater dynamic range to cover the large range of different concentrations of

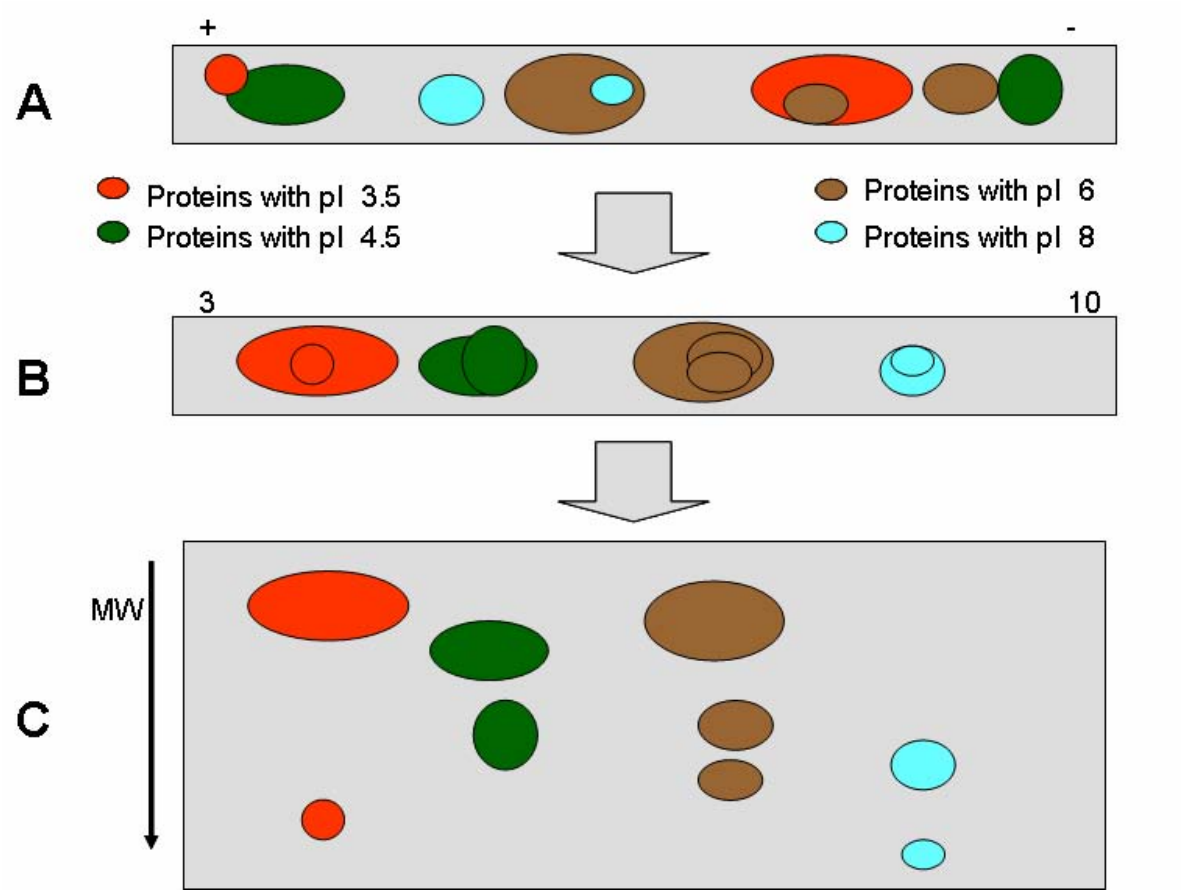

Figure 1. Two dimensional electrophoresis. (A). Before focusing, a mixture of proteins is loaded in a gel strip and is randomly distributed through the gel. (B). After focusing, proteins migrated in a electric field according to each protein pI's throughout a gradient pH of the gel strip (pH 3-10). (C). Focused proteins are separated according to their molecular weight (MW) in a SDS-polyacrylamide gel.

protein concentrations of protein in cell, protein turnover, and precise quantification of different proteins in order to understand the functional properties of cellular networks or to establish stoichiometries in multiprotein complexes.

Many different detection technologies have been developed in order to improve visualization of proteins in protein analysis. These include organic dye, silver staining, reverse stain, radiolabelling, fluorescent stain, chemiluminiscense and MS-based approaches. In addition to sensitivity, linear dynamic range and reproducibility, visualization methods should be fully capable of interacting with modern analytical tools for proteomics as image analysis softwares and different mass spectrometry methods. Image analysis software packages are intended to detect, capture, quantify and compare the relative value of each signal. Mass spectrometry is used according to the aims pursued: from the sole measurement of peptide masses of isolated proteins to identification of complex protein mixtures.

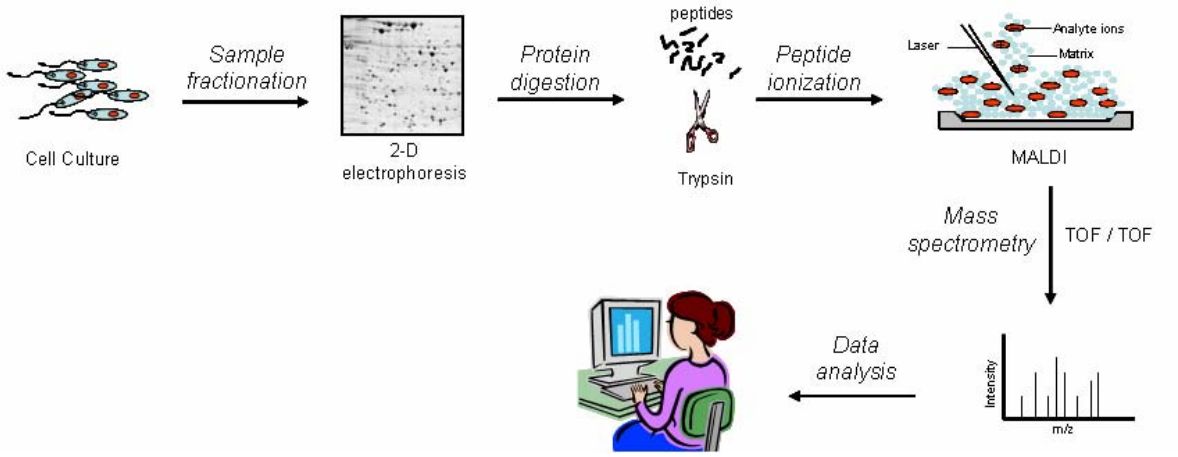

Figure 2. Proteomic experiment. Typical proteomic experiment conduced in the Department of Topical Medicine, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil. Proteins are purified from a cell culture and resolved in a 2-D gel. Proteins (spots) are cut from the gel and in-gel digested with trypsin. Peptides mixture are ionized by matrix-assisted laser desorption/ionization (MALDI) and analyzed by a time-of-flight mass spectrometer in tandem (TOF/TOF). Data obtained from mass spectra are searched against protein database using different search software.

A common strategy for protein detection is radiolabelling. Traditionally, protein radiolabelling is conducted *in vivo* (metabolic radiolabelling). Cells are labelled during their development or cellular cycle, allowing to follow the kinetics of synthesis and fate of specific proteins, and to track protein-protein and protein-nucleic acid interactions ranging from stabile complexes to transient contacts. Labelling of proteins from cells that came from specific media culture or cellular cycle is known as post-harvest labelling [4] and it is also used to improve detection and quantification of proteins. Usually a combination of radioisotopes in post-harvest labelling provides optimum detection of the entire proteome and high sensitivity to detect low copy proteins (Figure 3).

The measurement of radiotracers by scintillation counting has long been one of the most reliable methods for accurate, quantitative measurement in biochemical experiments. Detection of radiolabelled proteins separated by gel electrophoresis can be rapidly achieved using either autoradiography that offers a convenient, quick and cheap means of quantifying proteins, or if access to a machine is available, by using a phosphoimager. The isotope chosen must correspond to the question to be investigated but those in general use, either alone or in combination, are ^{3}H, ^{14}C, ^{32}P, ^{35}S or ^{125}I. Fluorography employs fluorescent enhancers impregnated into the gel matrix that improve detection of the radioactive emission through the generation of light. For high-energy isotopes, intensifying screens may be used to enhance the signal. These screens absorb the radioactive emissions that pass through the film, fluoresce and generate light that exposes the film. New technologies for detection of radiolabelled proteins, such as storage phosphor imagers and microchannel plate analyzers, offer a wider dynamic range and better sensitivity than an x-ray film. Labelling with radioactive isotopes of

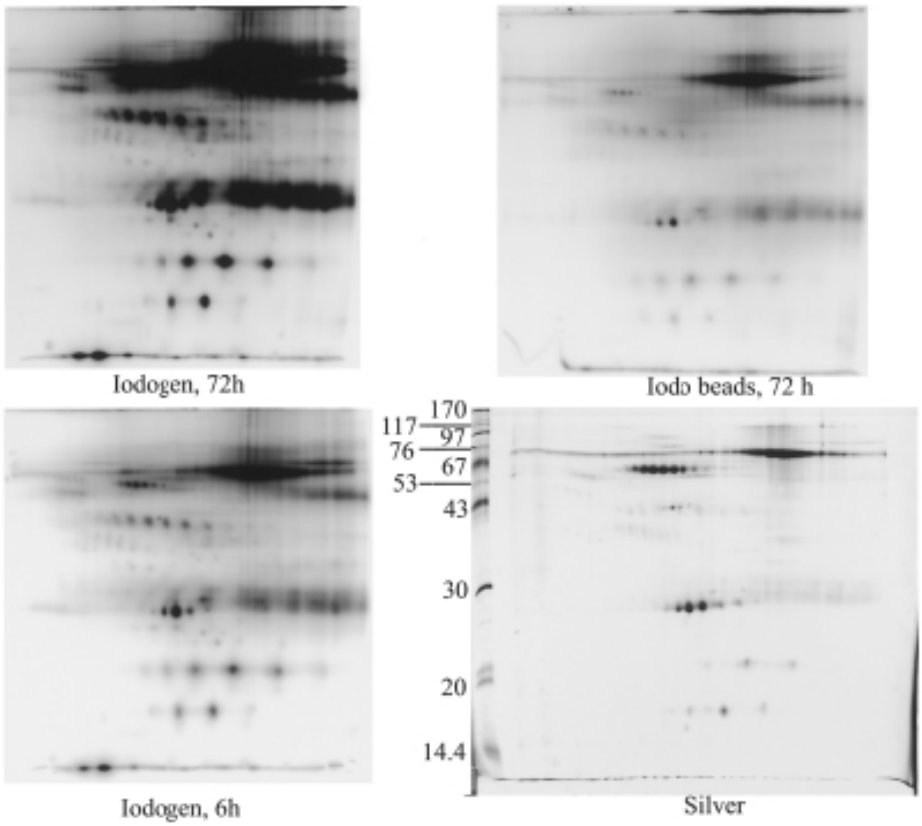

Figure 3. Comparison of sensibility of radioactive labeling (^{125}I) and silver staining in the detection of proteins separated by 2D gel electrophoresis. [Figure reproduced with permission of Dr. M. Cahill and Wiley-VCH Verlag GmbH & Co KG (Electrophoresis 2000, 21, 2594-2605)]

iodine, for example, allows detection of proteins down to the level of 1 attomole and lower, much more sensitive than conventional detection systems, and is able to quantify proteins over a linear dynamic range in access of 8 orders of magnitude. In post-harvest radioactive labelling of proteins, such improved sensitivity also has been demonstrated. For *in vivo* metabolic experiments, organic dye or silver stain methods are used in conjunction with radiolabelling techniques to simultaneously assess total protein expression levels along with protein synthesis rates, as well as to provide visual landmarks for the localization of the low abundance proteins in 2DE gels. An example of this is the study of Chen *et al*., (2004) [5] where chinese hamster ovary cells were metabolically labelled with ^{32}P and phosphoproteome was compared to general proteome stained with coomassie colloidal. The comparison of both proteomes revealed no correlation of intense phosphoproteins with intense colloidal spots, supporting the sensibility of specific radiolabelling.

Protein phosphorylation is a common post-translational modification of huge biological importance. This modification regulates a variety of essential biological phenomena such as enzyme activity, signal transduction, transcriptional regulation, cell division and differentiation, amongst others. The analysis of phosphoproteome is achieved by using ^{32}P for *in vivo* labelling phosphorylated proteins and further identification by MS. This approach allows the detection of very low abundance phosphoproteins below the limit of detection of the organic dye.

Currently, one of the major concerns in proteomic studies is the comparative analysis of 2DE gel images. Conventionally, two samples to be compared are separated on different gels. To search differences, each image is analyzed, spots are quantified, and then, identical spots between both gels are matched. However, accurate measurement of spot abundance remains a difficult task because variations exist between spot intensities even for identical spots on gels run in parallel. The spot matching is also difficult as protein patterns are never perfectly superimposable. The co-electrophoresis of fluorescence labelled samples arose to circumvent these problems. Because the proteins abundance from two samples is separated on the same gel, the comparison of spot is not dependent upon the reproducibility of 2DE gel system. In addition, the matching is facilitated as sample images are perfectly superimposable.

The differential gel exposure (DifExpo) method follows the same principle described above but introduces the use of radioisotopes to replace fluorescent dyes. Use of radioisotopes overcomes the drawbacks resulting from the use of fluorescent dyes. Cyanine dyes alter the migration of proteins on 2DE gels and labelling can be carried out only after protein extraction, limiting the possibilities of investigation. In DifExpo, the samples are differentiated from each other by the incorporation of two different isotopes ^{14}C and ^{3}H *in vivo* [6]. After cellular preparation extracts, the samples are submitted to 2DE, transferred to a polyvinylidene fluoride (PVDF) membrane and exposed successively to a storage phosphor plate sensitive to ^{14}C and to a plate sensitive to both ^{14}C and ^{3}H. After 70h exposure at 22°C, images are scanned in a phosphorimager and further processed with software to calculated specific spots measurements. ^{14}C intensity is obtained by measuring the peak height value of the spot detected on the ^{14}C plate. ^{3}H intensity is deduced from the peak height value of the corresponding spot detected on the $^{14}C^{3}H$ plate and the $^{3}H/^{14}C$ ratio of each spot is, then, calculated. The measuring of isotope ratio of each spot allows the detection of changes in the abundance of proteins present in cells at a very low level.

This technique has been used for comparison of cellular protein contents as well as for comparison of rates of protein synthesis. The synthesis of several yeast proteins is transiently induced in response to oxidative stress. A yeast culture was submitted to an oxidative stress, and proteins synthesized 20 to 35 min after the induction of the stress were labelled with [^{3}H]leucine. In parallel, proteins synthesized in the absence of oxidative stress were labelled for 15 min with [^{14}C]leucine. Determination of the $^{3}H/^{14}C$ ratio of 175 proteins observed revealed ten proteins markedly induced in response to oxidative stress [6]. The comparison of cellular protein contents is one of the first objectives of proteomic studies when two populations of cells are under study, or when one population is submitted to an insult condition. For this kind of comparison, DifExpo presents sensitivity comparable to silver staining. As mentioned above, DifExpo offers also the possibility of investigating changes in the rate of synthesis of individual proteins. By applying this technique it was possible to characterize yeast proteins transiently induced in response to oxidative stress as well as proteins repressed during the diauxic shift. When yeast cells grown on a glucose-based medium have exhausted their glucose supply, a transient growth arrest occurs. During this arrest, cells adapt their metabolism to the use of the ethanol produced during glucose fermentation. This phenomenon is called the diauxic shift [7]. The main drawback of DifExpo for investigating changes in protein synthesis lies in the low specific radioactivity of commercially available ^{14}C leucine and ^{3}H leucine.

Accelerator mass spectrometry (AMS) was originally developed around 1980 for highly sensitive radiocarbon dating, and that remains its predominant use in the 50 or so facilities worldwide [8-9]. AMS is typically used for determining the ratio of the abundant to rare

isotopes of beryllium, carbon, aluminum, chlorine, iodine and many others. It has the advantages of allowing the use of a smaller sample, faster analysis time and greater sensitivity than other mass spectrometry or decay counting techniques. So, AMS has the potential to quantify protein amounts to sufficient sensitivity and precision for proteome analysis. Some applications of this technique include the background quantification in 2DE gels [10]; protein identification for pharmacokinetics [11] and identification of covalent binding of labelled compounds to specific amino acids within an intact protein [12]. For proteomic studies [13], AMS promises to improve greatly the sensitivity of detection. Other commonly used staining technologies will miss low copy proteins (10-100 per cell) unless 20 mg of protein are analyzed with silver staining [12, 14].

A substantial improvement in the detection and quantification of radiolabels has recently been achieved with the development of multi-photon detection (MPD) methods. MPD methods are based on the coincident single particle detectors for measurement of multiple particles/photons in a single radioactive decay process (http: biotraces.com). The major advantage of the MPD methodology is its ability to detect certain radiolabels at levels below background radioactivity. The MPD imager has two identical detectors placed on each side of the gel. The detector consists of a position sensitive photomultiplier coupled with a CsI(Na) scintillating pixel array. In 2DE gel analyses, MPD offers gains in absolute sensitivity (zeptomoles), linear dynamic range (~10^7) and quantification of different proteins. Compared to previous multiplexing methods for relative quantification of proteins MPD has some advantages. By using single particle detectors, the sensitivity for detection of radiolabels can be improved dramatically; because single particle detectors can differentiate the particle energies produced by different decay processes, it is possible to choose combinations of radioisotopes that can be detected and quantified individually on the same 2DE gel; the MPD technology is essentially linear over 6 to 7 orders of magnitude, that is, it is possible to accurately quantify radiolabelling proteins over a range of at least 60 zeptomoles to 60 femtomoles. Finally, for radionuclides that decay by electron capture, *e.g.* with emission of both beta and gamma rays, co-incident detection of two particles/photons can be used to detect such radionuclides well below background radiation levels [15].

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Molecular genetic imaging using a nuclear medicine method

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Abstract. The remarkable efforts currently focussing on molecular nuclear technology signify its importance and wide range of application. With continued improvements in instrumentation, identification of novel targets, and design of better radioprobes, molecular nuclear imaging promises to play an increasingly important role in disease diagnosis and therapy. In the near future, through molecular imaging we can understand basic mechanisms of disease, and diagnose earlier and, subsequently, treat earlier intractable diseases such as cancers, neuro-degenerative diseases, and immunologic disorders. Iodine enters thyroid cells via a specific transporter, the sodium/iodide symporter (NIS), the gene of which was identified in 1996 [1]. The driving force of iodide uptake is the transmembrane concentration gradient of sodium ion, which is generated and maintained by sodium-potassium ATPase. NIS is an intrinsic membrane protein with 13 transmembrane domains. The recent characterization of the NIS protein and cloning of the NIS gene enable us to better understand the basic molecular mechanisms underlying iodide transport, and to clarify and expand its role in nuclear medicine. In addition, advances in molecular biology and its application in nuclear medicine have led to the development of novel radionuclide gene therapy and reporter gene imaging with results of translational bench works.

1. Introduction

Molecular imaging allows the visualization of normal and abnormal cellular processes at a molecular or genetic level rather than at an anatomical level [2]. In conventional images, the signal source is a nonspecific region of physicochemical interactions. However, the fundamental source of imaging signals is derived from a specific molecule or molecular event in molecular images. Molecular imaging is emerging as an exciting new discipline that deals with the imaging of disease on a cellular or genetic level. Moreover, because molecular and genetic changes precede anatomical changes during the course of disease development, molecular imaging can detect events that occur at the initial stages of disease progression.

2. Nuclear medicine and molecular imaging

There are three major means of molecular imaging: optical, MR, and nuclear medicine modalities (Table I) [2, 3]. Each imaging modality has several advantages and disadvantages. Optical imaging is highly sensitive and available. In vitro optical results are easily transferable to in vivo experiments. However, tissue attenuation of photons is a big problem when optical imaging is applied in large animals and humans. In addition, the system of tomographic images is not fully developed yet, and its low resolution is also a problem. Magnetic resonance imaging is fundamentally a tomographic system with a high resolution.

Table I. Methods of molecular imaging

1. Optical imaging
Fluorescence
Luminescence
2. Magnetic resonance imaging
3. Nuclear medicine imaging
Positron emitter
Gamma emitter

However, MR molecular imaging has low sensitivity and presents toxicity issues due to the use of iron particles. Nuclear medicine based molecular imaging has several advantages. It is highly sensitive and can detect at the 10^{-12} mol/L radiotracer level, and it is highly quantitative, which means that dynamic studies and kinetic modelling can be easily performed. Moreover, whilst optical imaging presents attenuation problems, nuclear medicine imaging has no such problems, which results in easy translation to human subjects.

Molecular imaging includes proteomic, metabolic, cellular biologic processes, and genetic imaging. Nuclear medicine has traditionally focused on the noninvasive physiologic imaging using radiolabelled tracers (Table II). For example, In-111 octreotide has been used for somatostatin receptor imaging, and radiolabelled antibodies for imaging antigen expressions in target cells. In particular, many Positron emission tomography (PET) tracers have been developed for imaging the biochemical metabolisms of normal and abnormal cells. Several biological cellular processes have also been visualized by molecular imaging. For example, Tc-99m labelled Annexin V and In-111 labelled arginine-glycine-aspartate (RGD) peptides have been developed for imaging apoptosis and angiogenesis. As such, molecular imaging has its roots in nuclear medicine and in many ways is a direct extension of this field.

Table II. Examples of Molecular Nuclear Imaging

Genomics: Radiolabeled substrates of HSV1-tk, D2R, NIS gene products
Proteomics
Receptor: In-111 octreotide
Antigen: In-111 anti-carcinoembryonic antigen antibody
Metabolism
Glucose: F-18 fluorodeoxyglucose (FDG)
Amino acid: C-11 methionine
Nuclear acid: F-18 fluorothymidine
Fatty acid: C-11 palmitate
Cellular biologic process
Differentiation: I-123, I-131
Specific function: Tc-99m sestamibi, neuroreceptor ligand
Apoptosis: Tc-99m annexin V
Angiogenesis: I-123 arginine-glycine-aspartate (RGD) peptide

3. Molecular-genetic imaging

In a narrow sense, molecular imaging means genetic imaging, so called “molecular-genetic imaging”, and currently, imaging reporter genes are usually used for molecular-genetic imaging. Conventional assays of reporter gene expression are useful for evaluating gene expression in cases of exogenous gene therapy. Initially, a fusion gene containing a therapeutic and a reporter gene is constructed and transfected into target cells. Thus, therapeutic and reporter gene transcription and translation occur simultaneously, and by assaying reporter gene protein products, the expressions of therapeutic target genes can be indirectly evaluated. Conventional assays for reporter gene expression are useful for evaluating molecular events. Common classic reporter genes, such as beta-galactosidase, alkaline phosphatase, luciferase, and green fluorescent protein, continue to play a critical role in the development of gene delivery systems for gene therapy. However, measurements of the expressions of these reporter proteins (enzymes) usually require tissue samples, and noninvasive and repetitive techniques are required to determine the transgene expression in

living animals. Imaging reporter genes produce reporter proteins, which induce the accumulations of specific imaging signals that facilitate the visualization of the locations, durations, and magnitudes of the transgene expressions. Thus, the imaging of reporter gene product activity levels by probe accumulation provides indirect information that reflects the level of reporter gene expression [4, 5].

3.1. PET system

Initially, imaging reporter genes were investigated in combination with high-resolution PET scanning. Current PET reporter gene imaging paradigms fall into two categories, i.e., the enzyme based (HSV1-tk gene) and the receptor based (dopaminergic receptor gene) methods [4-6].

The most commonly used is the Herpes Simplex virus type 1 thymidine kinase (HSV1-tk) gene method. Inside transfected cells, the HSV1-tk gene is transcribed to HSV1-tk mRNA, which is then translated to thymidine kinase (TK) enzyme. This TK enzyme then phosphorylates its substrate, for example I-124 labelled fluorodeoxyarabinofuranosyl uracil (FIAU). Unphosphorylated FIAU can traverse the cell membrane, but after phosphorylation, it cannot readily cross the membrane, and is thus trapped within the cell. I-124 labelled phosphorylated FIAU emits positrons that allow PET visualization.

HSV1-tk enzyme has two substrate types, F-18 or I-124 labelled thymidine derivatives like FIAU, and guanosine derivatives like acyclovir and ganciclovir [4-5]. PET images can localize reporter gene expression, and the magnitude of reporter probe accumulation in transduced cells reflects the level of HSV1-tk enzyme activity and level of HSV1-tk gene expression. The dopamine 2 receptor gene is also used for reporter imaging gene, because of the availability of the well established radiolabelled probe, F-18-fluoroethyl spiperone (FESP) [6]. Moreover, PET signals of F-18 FESP are well correlated with tritium labelled spiperone binding and D2 receptor gene expression.

3.2. Gamma camera system

However, conventional PET imaging reporter genes, such as HSV1-tk or D2R, require the synthesis of complicated substrates and the use of expensive PET equipment. A number of investigators have tried to use gamma emitters for molecular imaging [7]. Examples are somatostatin receptor 2 gene and In-111 octreotide, norepinephrine transporter gene and I-131 MIBG, and sodium/iodide symporter (NIS) gene and radioiodines and Tc-99m pertechnetate. Of these, the simplest and most applicable is the NIS gene system.

Other groups and we ourselves have proposed that the NIS gene may serve as an alternative imaging reporter gene to the conventional PET imaging reporter genes [8-10]. Iodide enters thyroid cells through a specific transporter with sodium; the so-called sodium/iodide symporter (Figure 1). Moreover, the driving force for iodide uptake is the sodium ion transmembrane concentration gradient, which is generated and maintained by the sodium-potassium pump (ATPase). This transporter co-transports sodium ion and iodide ion, thus the name sodium/iodide symporter.

The NIS gene was identified in 1996 by Dai et al. from rat [8]. The human NIS (hNIS) gene was also isolated and cloned using the complementary DNA sequence of rat NIS [9]. The hNIS gene contains 15 exons interrupted by 14 introns and codes for a 3.9 kb mRNA transcript. NIS is an intrinsic membrane protein with 13 putative transmembrane domains, an

extracellular amino terminal and an intracellular carboxy terminal domain. NIS protein has 3 potential N-linked glycosylation sites; one is located in the 7th extramembrane domain and 2 are located in the 13th extramembranous domain. NIS cotransport 2 sodium ions and 1 iodide ion and the transmembrane sodium gradient serves as the driving force for iodide uptake. In addition to iodide, several other anions are transported by NIS: $I^- = SeCN^- > SCN^- > ClO_3^- > NO_3^-$. NIS also transports Tc-99m and Re-188. The expression of functional NIS protein in target cell would enable cells to concentrate iodide from plasma, which offers the possibility of reporter gene imaging. NIS has many advantages as an imaging reporter gene due to the wide availability of its substrates, i.e., radioiodine and Tc-99m, and the well-understood metabolism and clearance of these substrates in the body. There is no problem of labelling stability when using radioiodine or Tc-99m, whereas this may be a major concern for the radiolabelled ligands of D2R and HSV1-tk. In addition, NIS is unlikely to interact with the underlying cell biochemistry. Iodide is not metabolized in most tissues, and although sodium influx may be a concern, no effects have been observed to date. Thus, reporter gene imaging may be easier with NIS, because all nuclear medicine departments have access to a gamma camera, radioiodines, and Tc-99m.

4. Applications of molecular-genetic imaging

Reporter gene imaging allows the visualization of the expressions of exogenous and endogenous genes, and of intracellular biologic events. Reporter gene imaging also can be used to monitor target cells, such as cancer, immune, and stem cells (Table III).

One major application of PET reporter gene imaging is the monitoring of the effects of gene therapy. PET imaging could be used to define the location, magnitude, and persistence of gene expression over time. Currently, experimental molecular imaging data are transferred to clinical practice. Jacobs et al. [10] reported the first human PET image of HSV1-tk expression in glioma patients. They performed HSV-tk suicidal gene therapy, and undertook to determine whether the gene was functionally active in human glioma cells. Before and after gene injection, they performed reporter gene imaging using radiolabelled FIAU, and confirmed the successful transfection of the HSV-tk gene into a human tumour *in vivo*.

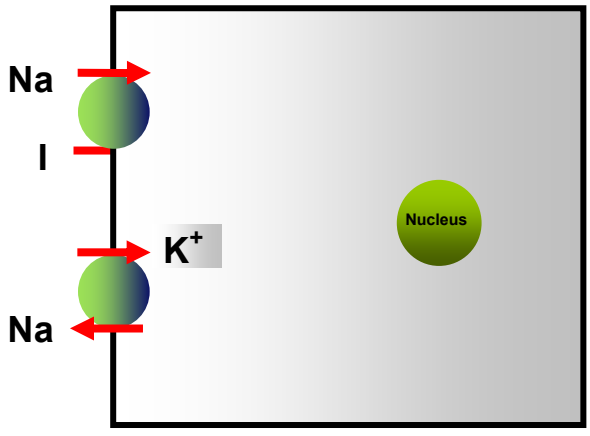

Figure 1. Sodium/iodide symporter (NIS). NIS is a cell membrane transporter which cotransports sodium and iodide ions into the cells.

Table III. Applications of reporter gene imaging

1. Gene expression
Exogenous gene expression
Endogenous gene expression
2. Intracellular molecular events
Signal transduction pathway
Activity of nuclear receptor
Protein-protein interaction
3. In vivo cell trafficking
Cancer cells
Stem cells
Immune cells

Several investigators have designed specific reporter gene constructs under the control of upstream promoter elements. These promoter elements can be sensitive to activation by specific endogenous transcription factors, and subsequently associate with specific endogenous genes. This strategy is referred to as the "*cis*-enhancer reporter gene system". Once a promoter has been activated due to the expression of an endogenous gene product, the reporter gene is also activated, enabling visualization using a nuclear medicine system. Thus, the increased expression of reporter gene within cells requires the increased activity of a particular promoter, which means an increased expression of the particular endogenous gene.

Some investigators have visualized the expression of the p53 gene using this *cis*-reporter gene system. Doubrovin et al. [11] used the HSV1-tk gene, and our group [12] used the NIS gene for reporter gene imaging, respectively. In our study, the p53RE-hNIS reporter system, in which the hNIS reporter gene is under the control of an artificial enhancer (p53RE), was constructed, and transfected into a human hepatoma cell line (SK-Hep1). Adriamycin was used to induce the expression of endogenous p53, and adriamycin treated cells accumulated more I-125 than non-treated cells. Moreover, I-125 uptake increased as the adriamycin dose was increased, and this correlated significantly with p53 levels determined by Western blotting with p53 specific antibody (Figure 2). Xenografted tumours of these cells also showed increased radionuclide accumulation after adriamycin treatment. Some studies have been conducted on specific endogenous gene targeting imaging of caspase, myc, and mdm genes.

Using imaging reporter genes, some intracellular biological events can be visualized. Blasberg's group imaged the intracellular signalling pathway of transforming growth factor (TGF)-beta receptor [13]. After the binding of TGF-beta to its receptor, a specific intracellular signal transduction pathway was found to be activated, which resulted in the production of several Smad proteins. They prepared a DNA construct containing a Smad binding promoter and the HSV1-tk-GFP imaging reporter gene. This DNA construct was the transfected into cancer cells, and a mouse experiment was performed. I-124 FIAU images, produced by administering TGF-beta, have visualized test tumours, indicating the presence of Smads, and successful signal transduction of TGF-beta into tumours. We also imaged the activities of the estrogens and retinoic acid receptors, using a *cis*-enhancer NIS reporter imaging system [14]. NIS and luciferase genes were linked with internal ribosome entry site (IRES) to express simultaneously two reporter genes, and placed under the control of a *cis*-acting retinoic acid

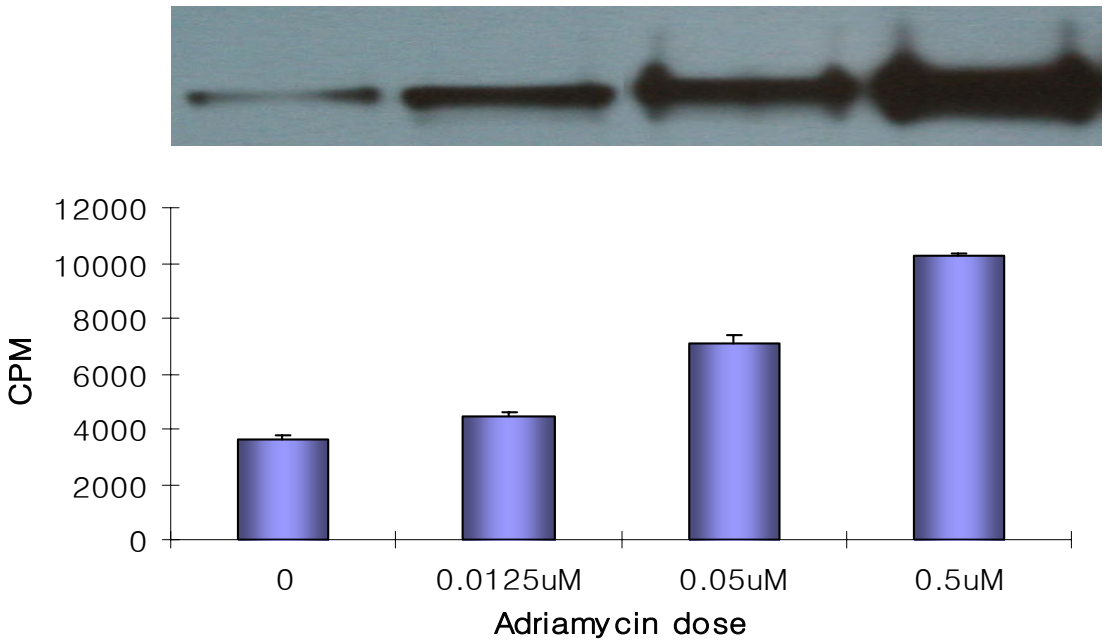

Figure 2. Relation between p53 gene expression and the accumulation of radioiodines in SK-Hep1 cells expressing p53RE-hNIS after adriamycin treatment. According to increased dose of adriamycin, increased expression of endogenous p53 gene and increased accumulation of radioiodine in the cells were observed.

responsive element (RARE). In human hepatoma cells containing this DNA construct, I-125 uptake, and bioluminescent intensity increased after retinoic acid treatment. The enhanced expression of NIS and luciferase gene by retinoic acid treatment was also demonstrated in an animal tumour model by scintigraphic images and optical bioluminescent images, respectively (Figure 3).

Molecular imaging using the NIS gene can be applied to the monitoring of the *in vivo* distributions of target cells, such as, cancer, immune, and stem cells. The hNIS gene under a constitutive promoter such as the Cytomegalovirus (CMV) promoter can be transfected to specific cancer cells. Using animal models, the effect of anticancer therapeutic regimens can be monitored easily by a gamma camera system using radioiodine or Tc-99m. We transfected the NIS gene into specific cancer cells, and found an excellent correlation (R^2=0.99) between accumulated radioiodine activity in cells and the number of viable cancer cells [15]. Reporter imaging using the NIS gene reflected viable cancer cell numbers, and allowed changes in cell numbers to be detected after anticancer treatment. *In vivo* imaging using Tc-99m showed a moderate correlation (R^2=0.78) between tumour weight and radioactivity. However, it is likely that radioactivity reflects viable cancer cell numbers more accurately than tumour weight, because tumour tissue also contains immune cells and necrotic and fibrous tissues. Koehne et al [16] demonstrated that Epstein-Barr virus (EBV)-specific T cells transduced with vectors encoding HSV-tk, selectively accumulate radiolabelled FIAU. After adoptive transfer of HSV-tk expressing T cells in Several Combined Immunodeficiency (SCID) mice, HSV-tk expressing T cells selectively accumulate in EBV specific tumours and can be noninvasively tracked by serial PET or gamma camera images. In this study, they monitored the migration of *ex vivo*-transduced antigen-specific T cells by nuclear medicine imaging

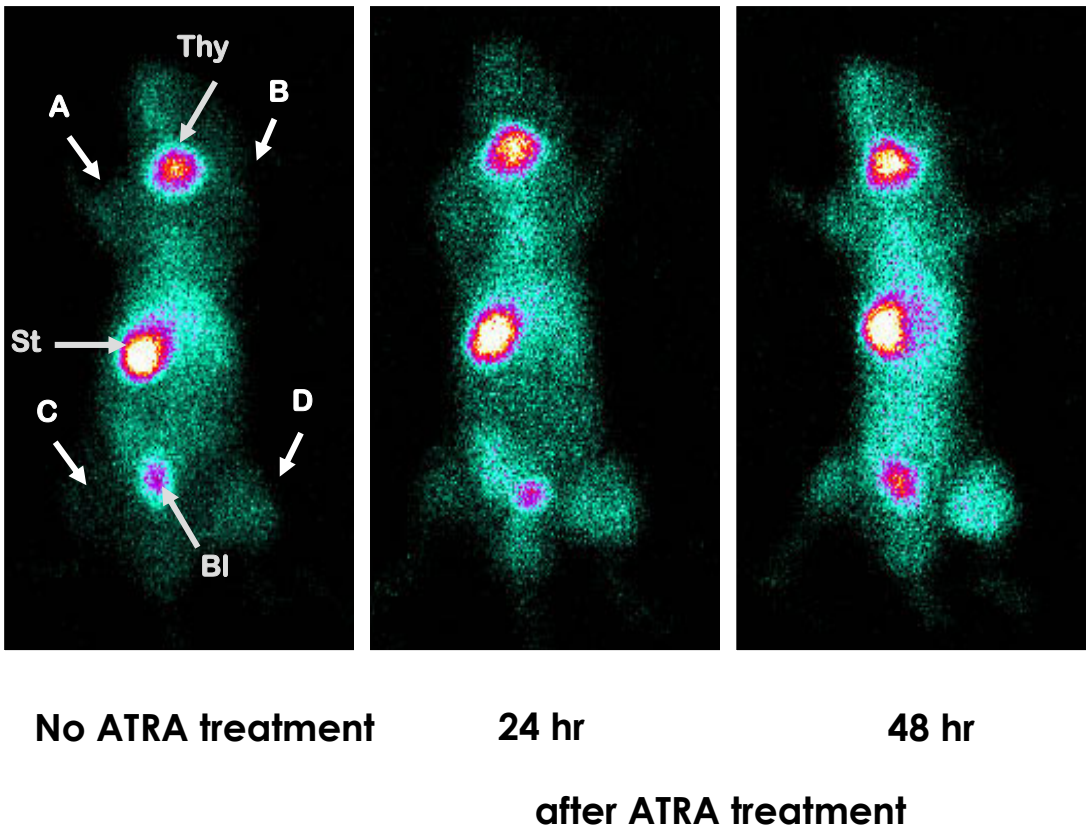

Figure 3. Scintigraphies using Tc-99m before and after treatment of all trans retinoic acid (ATRA) in a nude mouse bearing SK-Hep1 (A) and pRARE-NL expressing SK-Hep1 tumor (B, C, and D). (Thy ; thyroid, St; stomach, Bl; bladder). After ATRA treatment, increased accumulation of Tc-99m in the test tumors containing pRARE-NL was observed.

modalities *in vivo*. After the systemic or local injection of stem cells, stem cells and immortalized precursors may be able to migrate and repopulate ischemic sites in the brain or myocardium. The location(s), magnitude, and survival duration of embryonic cardiomyoblasts were monitored noninvasively using HSV-tk and a luciferase gene as the imaging reporter gene [17]. Moreover, stem cell migration can be imaged using NIS as an imaging reporter gene [18]. After the migration of stem cells to target tissue, differentiation to mature functional cells is critical for successful therapy. We have developed a transgenic mouse model to image cardiomyocyte differentiation using alpha-myosin heavy chain (MHC) promoter as a differentiation marker and NIS as a reporter gene [19]. Alpha-MHC promoter is known as a differentiated cardiomyocyte specific regulator for gene expression. A pMHC-hNIS construct comprising the NIS gene driven murine alpha-MHC promoter and a polyadenylation signal of bovine growth hormone was constructed. The linearized vectors were microinjected into fertilized eggs of FVB mouse strain, and ten positive founders were identified by PCR typing. I-131 dynamic scans of the positive founders showed rapid and intense myocardial radioiodine uptake. This transgenic mouse model should prove useful for myocardial stem cell differentiation studies. By injecting the bone marrow derived stem cells of these positive founders into animals with myocardial infarct, radioiodines or Tc-99m scintigraphy can be used to produce images of the differentiation of stem cells to mature cardiomyocytes.

5. Conclusion

The remarkable efforts that are currently focused on molecular nuclear technology demonstrate its potential importance and range of applications. Continued improvements in instrumentation, the identification of novel targets, and the availability of improved radioprobes indicate that molecular nuclear imaging is likely to play an increasingly important role in disease diagnosis and therapy. However, it should be mentioned that dual or triple reporter constructs embracing optical, MR, and nuclear medicine provide opportunities for multi-modality imaging, which should ease the translation of laboratory studies to the clinical level. In the near future, molecular imaging will undoubtedly increase our basic understanding of disease mechanisms, and facilitate earlier diagnoses and treatment of intractable diseases like cancers, neuro-degenerative diseases, and immunologic disorders.

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Minimal residual disease testing with radioisotopes

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Abstract. The aim of any therapy is to eradicate minimal residual disease (MRD) by studying the efficacy of different regimens. It allows us to design more aggressive therapy. It allows us to evaluate novel drugs, which target cellular mechanisms such as differentiation agents and apoptosis inducers and inhibitors. MRD is necessary to evaluate the efficacy of comparative treatments. It may be used to select patients and determine when and whom to treat. In the bone marrow transplant setting it may be used to assess the purity of the donor cells and to track the donor cells following transplantation. It allows the assessment of relevance of maintenance therapy. There are several techniques for detecting MRD with differing sensitivities. Cytology is very much the gold standard with haematologists and a leukaemia cell can be detected in 100 cells. In vitro progenitor assays involving the growth of cells [colony forming units (CFU)] following cytokine addition [myeloid progenitors include CFU-GM (for granulocyte-macrophage), CFU-G (for granulocyte), CFU-M (for macrophage); erythroid colonies are called burst forming units (BFU-E) and lymphoid colonies] have a similar sensitivity. Immunophenotyping using a panel of surface markers and flow cytometry can detect 1 abnormal cell in 10,000. Conventional cytogenetics by Giemsa staining can detect 1 in 30 cells. Using fluorescence is situ hybridisation on metaphase chromosome spreads or interphase nuclei increases the detection rate to 1 in 100 cells. By far the most sensitive of techniques is the polymerase chain reaction (PCR) based assays. PCR is highly specific, very sensitive, quantifiable, and can be standardized and automated. PCR has a sensitivity of 1 in 10^5-10^6. Reverse transcriptase-PCR (RT-PCR) using conventional reactions have sensitivities of 1 in 10^4-10^6 whereas real time PCR has a detection rate of 1 in 10^4-10^5. PCR followed by Southern blotting and hybridisation with radio-labelled probes are even more sensitive at 1 in 10^5-10^7 with detection of transcripts as low as 1fg [1]. Radioactivity provides for very sensitive in situ hybridisations [2]. For solid tumours imaging systems such as magnetic resonance imaging (MRI), computed tomographic (CT) and positron emission tomography (PET) is very useful for tracking disease bulk. Of these methods PET and the combination of PET-CT give the best results.

1. Mutations

Many abnormalities occur in genes, which control cell maturation, proliferation or programmed cell death. The candidate genes to follow are numerous (Figure 1). They involve gain of function as well as loss of function mutations. The genes are localized in all parts of the cell. One can detect point mutations simply by PCR and oligonucleotide hybridization with a specific $^{32}P\gamma ATP$ labelled probe (Figure 2). We have been studying 4 candidate genes in pre-leukaemia namely the signal transducer RAS, the colony stimulating factor – 1 receptor (FMS), the tumour suppressor gene p53 and the differentiation specific retinoic acid receptor alpha (RARα). We have followed a cohort of 75 patients over 10 years assayed for mutations and shown that patients with mutations do badly compared to patients without mutations with a median survival of 13 months in the former compared with 30 months in the latter (Figure 3). Furthermore, of the 20 patients who progressed to AML, 14 of them harboured a RAS mutation [3-6]. We have also detected RAS and FMS gene mutations in previously treated patients who were in remission from childhood leukaemia or lymphoma and at risk of developing secondary leukaemia. These patients have low frequencies of RAS mutations (6-16%) [7, 8]. More recently techniques using denaturing high-performance liquid chromatography (DHPLC) or Nucleic Acid Fragment Analysis System (WAVE®) have been used for detecting point mutations (Figure 4). This technique has a sensitivity of 5%. In other words it can detect mutant bearing cells if they constitute at least 5% of the total population. FLT3 abnormalities have also been associated with poor prognosis in AML (Figure 5) [9-11].

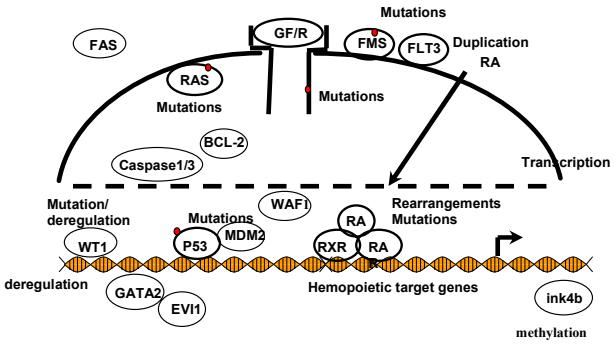


Figure 1. Genetic lesions in leukaemia; candidate oncogenes in malignancies.

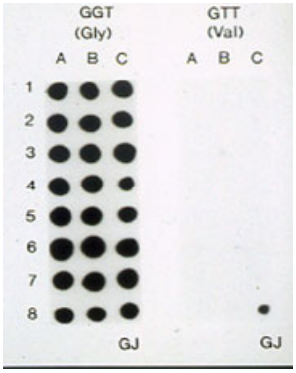
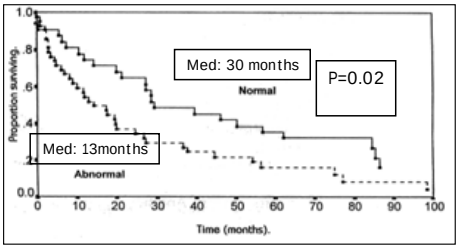


Figure 2. RAS mutations in myelodyslpastic patients. Amplified DNA from bone marrow of MDS patients were dot blotted and hybridised with 32P-γATP – labelled oligonucleotide probe specific for wild type NRAS12(Gly) and mutant specific NRASV12(Val. Reproduced from the author's papers with kind permission from Leukemia [3,4].

75 preleukemic patients followed for 10 years (1987-1997)
50% patients had at least one mutated gene

Gene tested	RAS	FMS	p53	RARα
No. pts with mutations (No. pts tested)	36(75)	9 (75)	8 (50)	6 (153)
%	48%	12%	12%	2%

Candidate genes for survival (n=75)



Candidate genes for transformation to leukemia

20 AML

- **RAS 14 (70%)**
- **FMS 3**
- **p53 0**
- **RARα 0**

Figure 3. Gene mutations result in a poor prognosis. Mutations of RAS, FMS, p53 and RARα were detected in 50% of the MDS patients. Kaplan-Meier survival curves showing decreased survival in patients harbouring mutations and increased chances of progression to AML [3-6]. Kaplan-Meier survival curve reproduced from author's publication with kind permission from Leukemia [3].

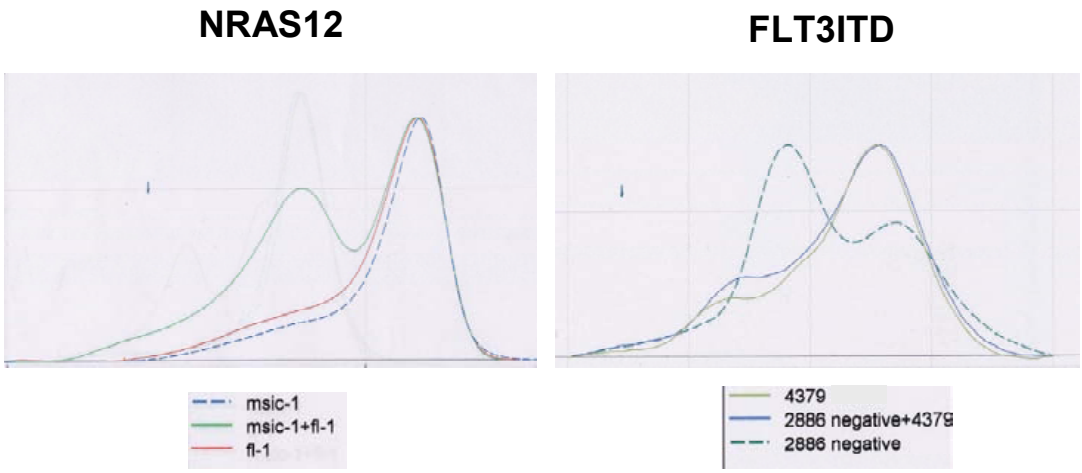


Figure 4. Detection of mutations by WAVE. Amplified DNA was analyzed by heteroduplex analysis using a DHPLC based method (WAVE). The heteroduplex indicative of mutation elutes faster than the homoduplexes. Mutations around codon 12 of NRAS and FLT3 internal tandem duplications were detectable [Morgan & Padua, unpublished].

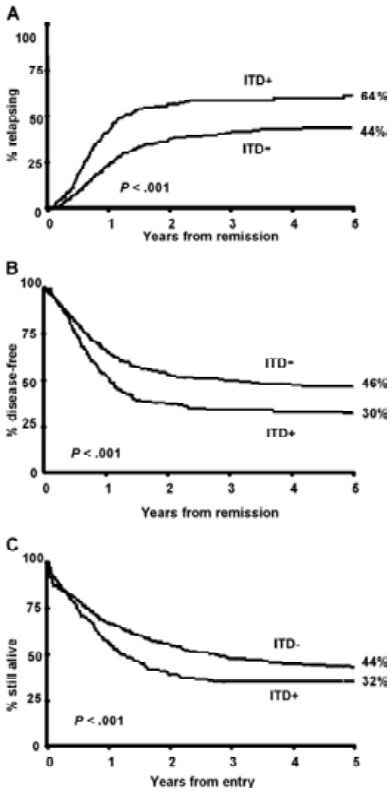


Figure 5. Kaplan-Meier curves for AML patients with (ITD+) or without (ITD-) a FLT3/ITD. A) RR, B) DFS, C) OS [10; Reproduced with kind permission from Rosemary Gale and publisher's of Blood].

FLT3 inhibitors have been shown to have efficacy in FLT3 driven childhood lymphocytic leukaemia [12]. Other genes currently under evaluation for diagnostic and prognostic utility by the working party 12 (WP12) of the EU leukaemia network include mutations of WT1.

2. Translocations

Gene fusions have been detected by cytogenetics occuring in around 50% of all leukaemia [13], and in myeloid leukaemia the PML-RARα (t(15;17)), AML1-ETO (t,(8;21)) and CBFβ-MYH11 (inversion16) fusions are associated with a good prognosis in AML (Figure 6) [14, 15]. These fusions can be tracked and quantified using real time RT-PCR techniques [16] (Figures 7A & B). With the PML-RARα fusion there are 3 breakpoints and RT-PCR can be undertaken to identify them. The type of break point has predictive value as patients with the break point cluster region 1 (bcr1) have a better survival than patients with bcr2 or bcr3 breakpoints (Figure 8, Cassinat, unpublished).

Targeted therapies have come of age since the use of *all-trans* retinoic acid (ATRA) for APL and Glivec for CML [17]. The advent of Glivec, a tyrosine kinase inhibitor, has revolutionized the clinical management of chronic myelogenous leukaemia (CML). RT-PCR has been crucial for monitoring MRD in patients bearing the major break point cluster region (BCR), which maps on chromosome 22 and the ableson gene (ABL), which originally mapped on chromosome 9. The translocation of ABL sequences to BCR creates a fusion gene on the Philadelphia chromosome (Ph') [18]. Many patients remain positive for the BCR-ABL fusion transcript created by the t(9;22) translocation. What has been informative was the log drop in transcript levels following treatment. In our hands conventional RT-PCR has a sensitivity of 1 in 10^4 (Figure 9, Nickless & Padua, unpublished). However, drug resistance can be a problem due to point mutations arising in the ATP binding domain. Mutations can be assayed by WAVE (Figure 10, Morgan & Padua, unpublished). Patients with persistent mutations remain drug resistant and dose escalation may be warranted. Emerging technologies of gene profiling can also be used to predict drug resistance [19]. These strategies will allow treatment to be modified. New compounds such as Dasatinib, which is a multi-targeted kinase inhibitor of BCR-ABL and SRC kinases, which also bind the ATP pocket are in ongoing clinical trials to overcome Glivec resistance. However, these patients develop a different spectrum of mutations, which can be monitored by WAVE.

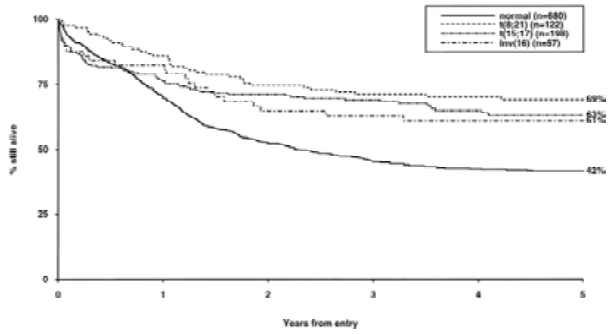
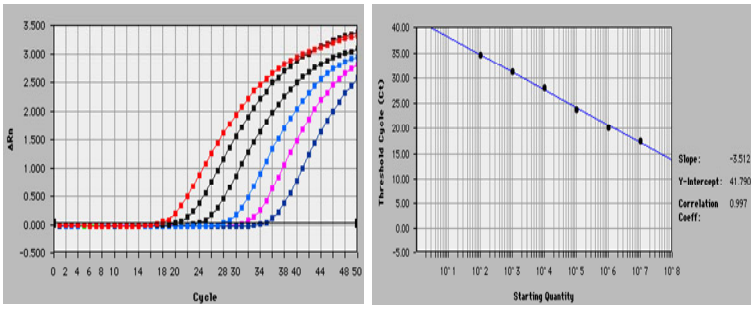
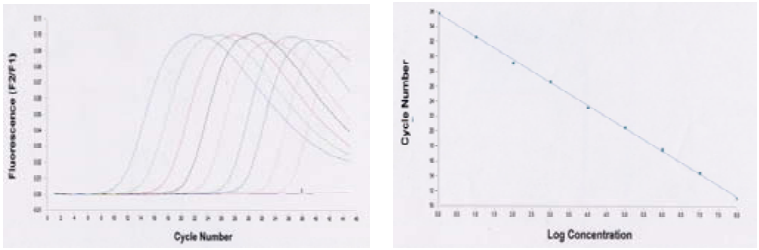


Figure 6. Overall survival of patients with favorable cytogenetic abnormalities, irrespective of the presence of additional abnormalities. The group with normal karyotype is included for comparison. [14; Reproduced with kind permission from David Grimwade and publishers of Blood].



A) TaqMan technology (Robin & Padua, unpublished)



B) Light Cycler technology (Morgan & Padua, unpublished)

Figure 7. Real Time RT-PCR. RT-PCR of PML-RARα showing increasing amounts of transcripts requiring decreasing numbers of cycles. The data were generated with control PML-RARα plasmids. Using the linear graph one can determine the copy number of the test sample by reading the number of cycles required to detect the transcript and reading the log concentration directly. Attempts to standardize these assays are being undertaken by the WP12 of the EU Leukemia Net. Plasmids are being distributed to laboratories as both TaqMan and Light Cycler platforms are in use.

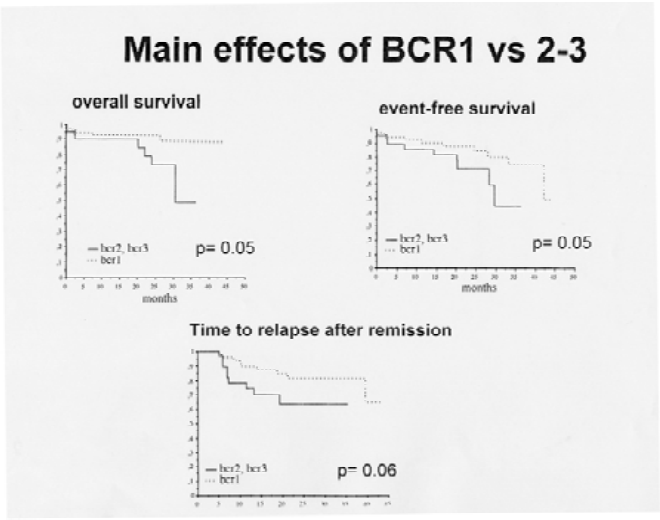


Figure 8. PML-RARα breakpoints and survival. There are 3 breakpoints depending on where in the PML gene the fusion breaks (bcr1, bcr2 and bcr3). The majority of patients (70%) have the bcr1 breakpoint and do well whereas patients with bcr2 and bcr3 breakpoints have a poor prognosis and require further treatment and careful MRD monitoring (Reproduced by permission from Cassinat, unpublished).

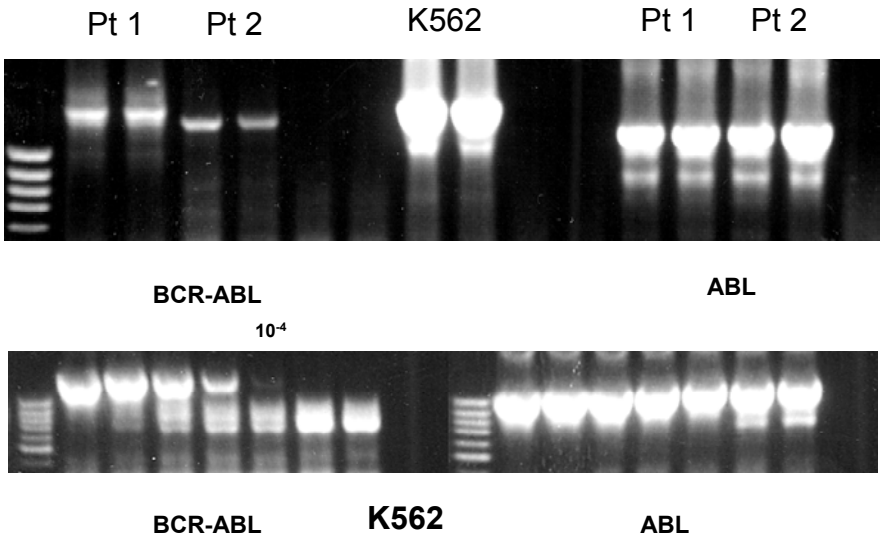


Figure 9. RT-PCR of BCR-ABL transcripts demonstrating the sensitivity of the assay. RT-PCR of BCR-ABL bearing K562 cell line and chronic myelogenous leukemia (CML) patients (Pt1 and Pt2) with corresponding ABL RT-PCR. The reactions were set up in duplicate Reconstruction experiments with successive 10-fold dilution starting with undiluted and diluting down to 10^{-6} show that BCR-ABL transcripts can be detected with a sensitivity of 1 in 10^4 (10^{-4}) (Nickless & Padua, unpublished).

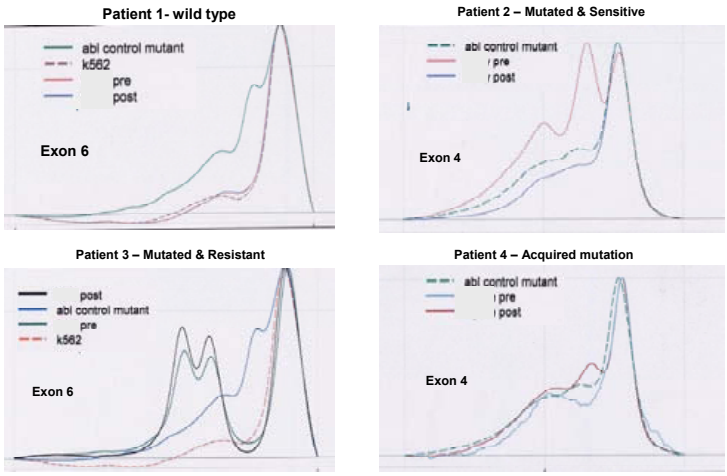


Figure 10. ABL mutations detected by WAVE.
RT-PCR products of RNA from CML patient and the K562 cell line analyzed by heteroduplex and WAVE. Different responses to Glivec treatment are shown with WAVE patterns from paired pre and post treatment samples. Patient 1 does not have a detectable mutation before (pre) or after (post) treatment. Patient 2 has a detectable mutation pre treatment, which is undetectable post treatment. Patient 3 has a detectable mutation pre and post treatment. Patient 4 does not have a detectable mutation pre treatment, but has a detectable mutation post treatment, which may have been acquired or selected for. (Morgan & Padua, unpublished).

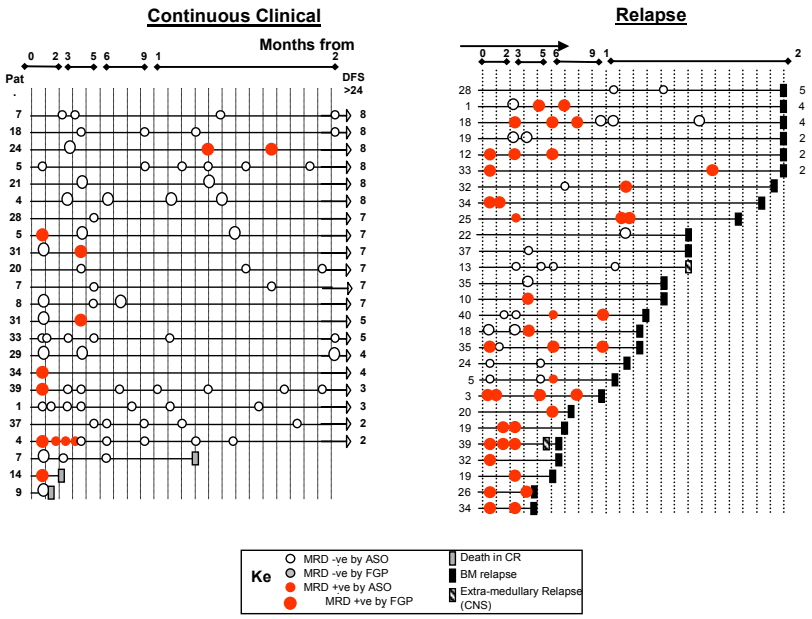
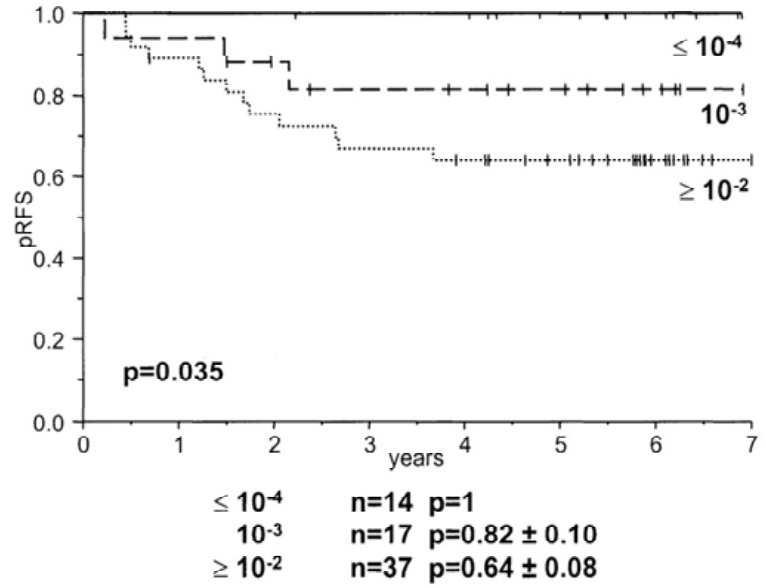


Figure 11. Adult B lineage ALL treated with chemotherapy (50 patients). Minimal residual disease (MRD) testing by polymerase chain reaction (PCR) correlates with disease outcome. CR= complete remission. BM= bone marrow. CNS= central nervous system. Patients who were persistently MRD positive (in red) were prone to relapse [20]. (Reproduced with kind permission from Letizia Foroni and publishers of Best practice Research in Clinical Hematology).

Acute lymphoblastic leukaemia (ALL) similarly has gene fusions, which have been extensively studied for MRD using PCR based techniques (Figure 11) [20, 21]. Based on their PCR status, adult ALL patients can be grouped into patients in clinical remission with PCR negativity and relapsed cases, where patients remain PCR positive. Current treatment for childhood ALL results in an overall relapse free survival of approximately 75% [22, 23]. Leukaemia clone-specific antigen receptor gene rearrangements (IgH, Igk, TCRD, TCRG) can be amplified from DNA of diagnostic bone marrow by PCR. Junctional region-specific radioactively labelled oligonucleotide probes on dot blots of amplified DNA can detect residual disease. Using these methods MRD testing of childhood ALL has enabled the risk stratification of patients resulting in individualising treatment protocols [21, 24, 25]. In childhood ALL risk stratification based on day 15 post treatment MRD testing is possible (Figure 12A) [26]. Patients with transcripts detectable at 1 in 10^2 do badly, with those with transcript levels detectable at 1 in 10^4 doing best. By day 28, patients can be stratified into low, intermediate and high-risk groups based on their MRD determined by PCR (Figure 12B) [26] Thirty years of clinical trials in childhood ALL have brought us to a point where we can predict the outcome of treatment in individual patients and tailor treatment to maximise cure while minimizing toxicity [27].

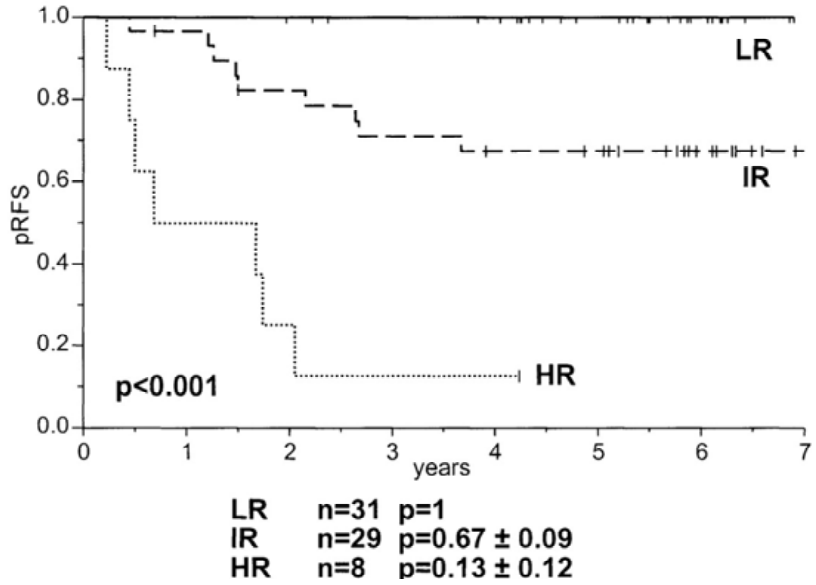
3. PET (positron electron tomography)

Many of the PCR methods for MRD use fluorescent probes and machines, which amplify and quantify DNA/RNA in real time. Radioactivity is still used routinely in immunological tests to measure proliferation (tritiated thymidine ^3H incorporation) and cytotoxic T-cell kill (Chromium (Cr) release assay). However, non-radioactive methods are



A) Probability of RFS according to day-15 MRD levels

Patients with less no. of transcripts (< 1 in 10^4) have a better relapse free survival (pRFS) than patients with higher transcript levels (1 in 10^2).



B) Probability of RFS according to MRD-based risk group stratification.

Risk stratification based on day 15 and day 28 MRD testing. LR = low risk, IR = intermediate risk, HR = high risk.

Figure 12. Risk stratification based on MRD testing [26]. (Reproduced with kind permission from Dr Panzer Grumayer and the publishers of Blood).

used where radioactivity plays a major role. It is used for pre-treatment staging, in remission assessment and determining prognosis for solid tumours. Positron emitting isotope 18-fluorine fluorodeoxyglucose (FDG) is preferentially accumulated and trapped within tumour cells, to provide high quality and high-resolution (3mm) whole body images. In Hodgkin's disease (HD) and Non-Hodgkin's Lymphoma (NHL) the images are of nodal and extra nodal disease. FDG-PET has become an indispensable tool for the management of lymphomas, where accurate anatomical assessment of disease distribution is vital for planning curative treatment of several subtypes of lymphoma (Figure 13 for examples) [28]. PET provides more accurate staging than CT, MRI, Gallium and SPECT scanning (Figure 14).

In comparisons of PET with CT for lymphoma staging at diagnosis, a high degree of concordance in identifying nodal disease is shown. PET is more sensitive for detecting disease in small or borderline enlarged lymph nodes. PET has a high detection rate of extra-nodal disease without biopsy (for example in the liver, lung, kidney and bone marrow). It increases CT based Ann Arbor disease stage in 15-20% of HD and NHL patients.

PET is particularly valuable for assessing disease clearance or persistent residual disease in lymph node masses identified by CT (Table I) and response to chemotherapy (Figures 10-13). It is also useful in assessment of the sites of relapse (Figure 15). Early monitoring by PET can predict long-term outcome. HD and high grade NHL are usually treated with 6 cycles of chemotherapy. When the disease status is assessed by PET after 2 cycles of chemotherapy the 2.5 year relapse rate for PET+ patients is 87% and for PET negative patients 0% [29, 30] (Figures 16-18).

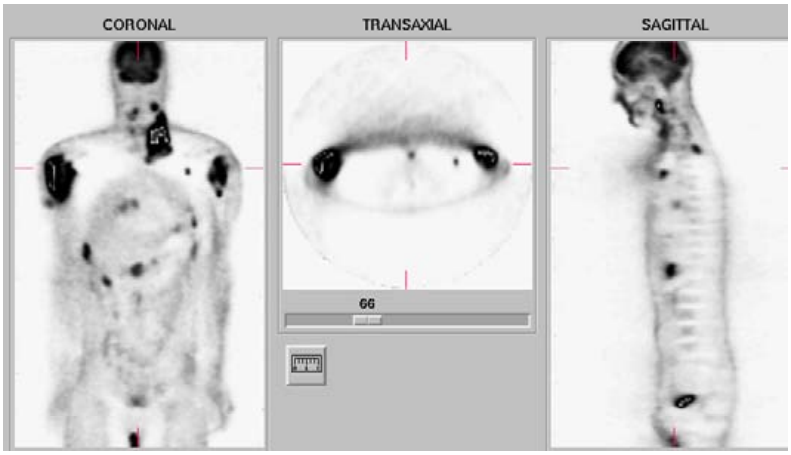


Figure 13. Staging of disease with PET. 29M HIV with cervical node- biopsy showed "reactive node". PET shows extensive disease in the neck, axillae, proximal humeri, mediastinum, hila, liver, lung, under the diaphragm. Re-biopsy of PET positive LN group confirmed diagnosis of High Grade NHL. Reproduced from the teaching archives of the Guys King's and Thomas's Medical School provided by Thomas Nunan.

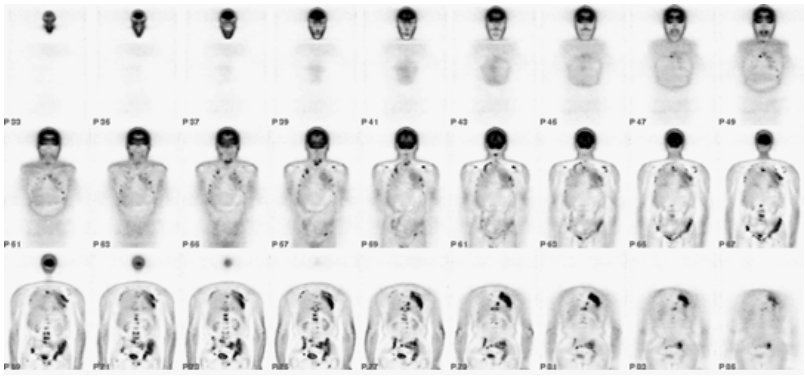


Figure 14. Staging of disease. 52M Diffuse large B cell NHL on biopsy of left axilla. Clinical stage I or IIa. Staging CT shows only 0.7cm node in left neck. PET reveals extensive disease both above and below the diaphragm, including patchy bone marrow involvement (note "hot" thoracic and lumbar vertebrae) - Stage IV disease requiring different treatment approach. . Reproduced from the teaching archives of the Guys King's and Thomas's Medical School provided by Thomas Nunan

Table I. Relapse and rate related to post retuximab-imaging assessment.

Imaging method	Positive	Negative	Significance
CT	41%	25%	p>0.1
PET	100%	18%	P<0.001

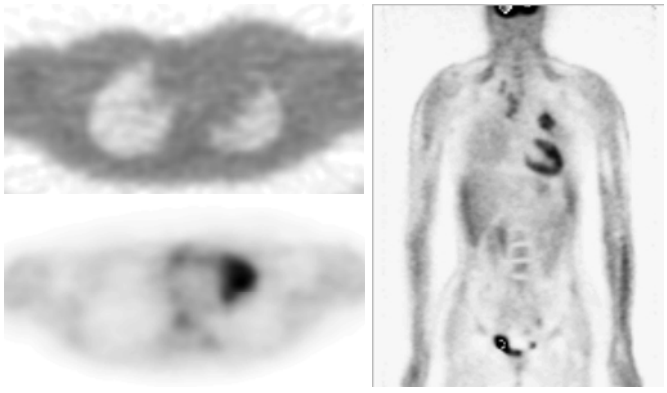


Figure 15. Assessment of residual masses. 14 y.o. female Hodgkin's Disease, treated with chemotherapy. MRI (top left panel) post therapy showed mediastinal mass. PET - residual disease in the mediastinum. Reproduced from the teaching archives of the Guys King's and Thomas's Medical School provided by Thomas Nunan.
Further treatment given: mediastinal mass subsequently became PET negative

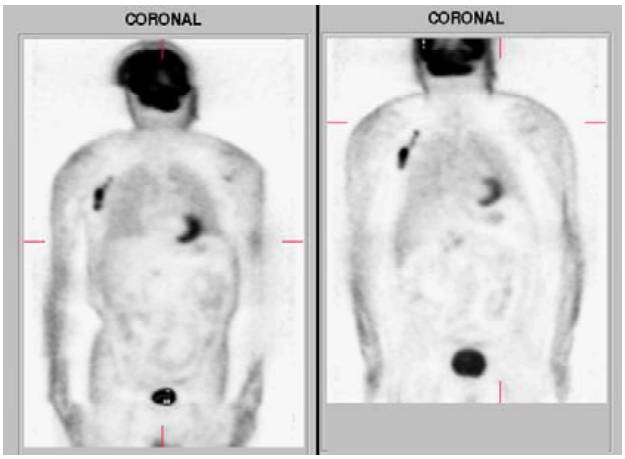


Figure 16. Response to chemotherapy. Partial remission after 6 cycles of chemotherapy. Scanned after 2 further cycles. Palpable axillary adenopathy persists, which may be scar tissue or persisting disease. The PET scan shows persistent, but localized disease after full chemotherapy treatment. The patient was given local radiotherapy, with a good response. Reproduced from the teaching archives of the Guys King's and Thomas's Medical School provided by Thomas Nunan.

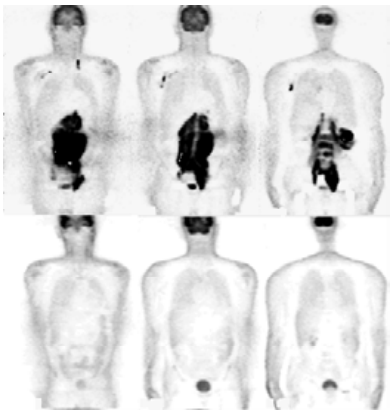


Figure 17. Response to chemotherapy. Middle aged male with non-Hodgkins lymphoma. Pre treatment PET shows extensive disease above and below the diaphragm. Interim scan, after 2 courses of chemotherapy, shows complete resolution of the changes. Suggests good prognosis, in spite of extensive disease at presentation. Reproduced from the teaching archives of the Guys King's and Thomas's Medical School provided by Thomas Nunan.

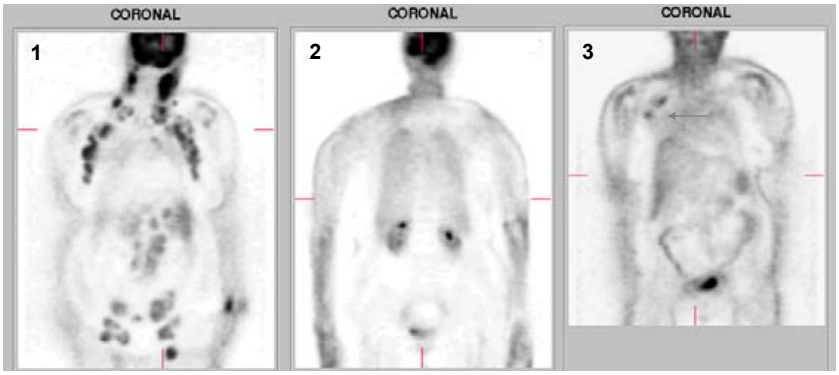


Figure 18. Assessment of sites of relapse. Pre-treatment extensive disease (panel 1). Post Chemotherapy PET showed complete response (panel 2). Recurrent axillary nodes, CT shows nodal enlargement: possible early relapse, PET positivity confirms disease recurrence, without need for biopsy (panel 3). PET January, March & July. Reproduced from the teaching archives of the Guys King's and Thomas's Medical School provided by Thomas Nunan.

4. Chimerism

In the bone marrow transplant setting, MRD can be assessed by tracking donor cells in an allogeneic transplant where the donor cells come from another individual, usually a matched sibling or a matched unrelated donor (MUD). Full ablative conditioning with chemotherapy prior to bone transplant is only possible in relatively young (<55 years old) patients and carries a high mortality due to the procedure. Allogeneic transplants using reduced intensity conditioning (using lower doses of chemotherapy and in addition in some protocols using Campath or alemtuzumab, an antiCD52 antibody) has enabled the recruitment of elderly leukaemia patients to bone marrow transplantation with some success [31, 32]. Using multiplex PCR of 16 genes, which are highly polymorphic, including a sex mismatched gene, which maps to the X chromosome, chimerism of different lineages to track donor and recipient haematopoiesis after bone marrow transplantation has been highly predictive of relapse. The rate of engraftment of different lineages and kinetics of relapse allows for early intervention. The CD3 peripheral blood fraction is particularly predictive as it is the first to lose the graft (Figure 19). This allows the precise timing of further donor lymphocyte infusions. These investigations can be supplemented by mismatched FISH (fluorescent in situ hybridization) for sex-mismatched transplants (Figure 20). These tests have been used for routine patient management.

5. Gene profiling and discovery

Gene profiling has been extensively used to search for prognostic markers for accurate diagnosis, for predicting response to treatment and to find novel targets for therapy. This technique involves spotting sequences, in the form of either clones, amplified cDNAs or synthetic oligonucleotides on slides representing thousands of genes covering the entire genome or in some cases more restricted sets to cover particular functions such as proliferation or cell cycle, apoptosis or programmed cell death, differentiation or maturation. The test sample is then prepared by extracting RNA, and cDNA from these samples are made by reverse transcriptase and later hybridised in turn to the panel of genes. The analysis of

these images called dendrograms can be varied. Normally red colours show high expression, going down to yellow, which usually means no change in expression, to blue or green to denote downregulation. The analysis can be undertaken in functional groups, such as apoptosis or programmed cell death genes, cytokines or growth factors genes and distinctive signatures can be observed for specific diseases and subtypes of diseases.

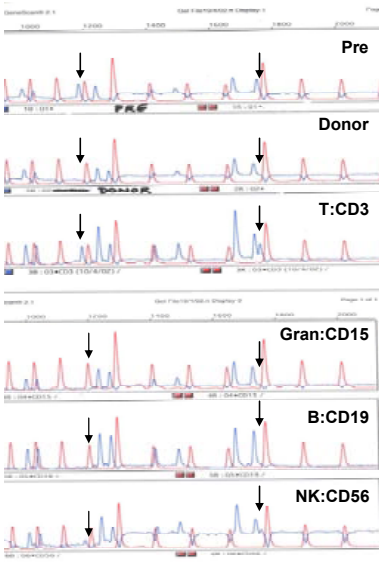


Figure 19. Short tandem repeat PCRs showing chimera post bone marrow transplant. Amplified products of recipient pretransplant (pre), donor and peripheral blood DNA extracted from cells separated by an AutoMacs (Miltenyi). Fluorescent-labelled PCR products are fractionated on acryl amide gels and fluorescence is scanned. The CD3+ and CD56+ showed a mixed chimeric pattern, whereas the CD15+ and 1+9 fractions show full donor chimerism (Pearce & Padua, unpublished).

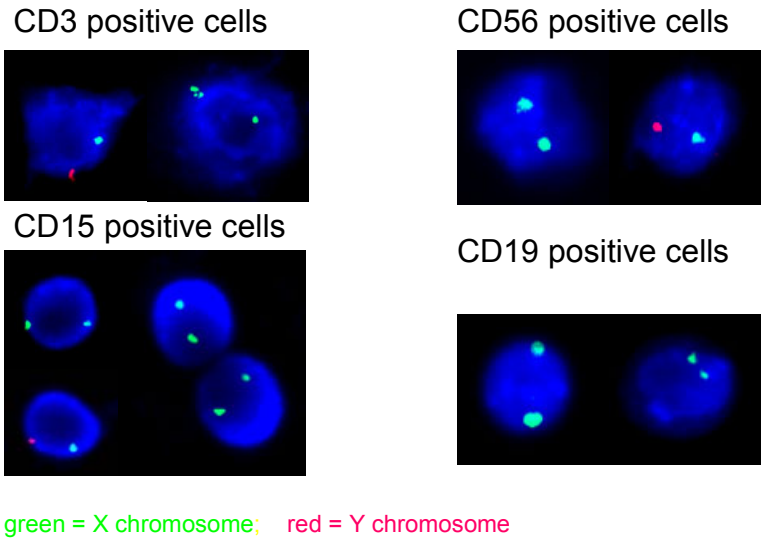


Figure 20. Lineage specific XY FISH. Interphase nuclei from peripheral blood cells separated using an AutoMacs and probed with fluorescent chromosome X-specific (in green) and chromosome Y-specific (in red) probes, confirming the short tandem repeat results (Adams & Padua, unpublished).

One of the best studied is the use of the technology in lymphoma. Distinct types of diffuse large B-cell lymphoma (DLBCL) has been identified (Figures 21-22) [33]. These profiles split the patients into distinct prognostic groups. Using these arrays and those generated by

other groups and reanalysis Lossos et al. [34] proposed a model based on 6 genes, which would predict survival in diffuse large-B-cell lymphoma (Figures 23). The genes with the strongest predictor are *LMO2, BCL6, FN1, CCND2, SCYA3* and *BCL-2*. Three of these genes were down-regulated (*LMO2, BCL6, FN1*). *LMO2*, is involved in erythropoiesis and angiogenesis is the site of a chromosomal translocation in childhood leukaemia. *BCL6* is a transcription factor and *FN1* for fibronectin 1 is an extracellular glycoprotein, the ligand for an integrin family member. Integrins are cell adhesion molecules whose upregulation is likely to be associated with metastatic disease. Upregulation of BCL-2, an apoptotic gene originally isolated at the breakpoint of a non Hodgkin's lymphoma (NHL) with a t(14;18) translocation, *CCND2*, a cell cycle regulator belonging to the cyclin family and *SCYA3* or *MIP1alpha*, a chemokine which recruits a variety of cells to sites of inflammation all correlated with a short survival. In a proteomics study expression of the *FOXP1* transcription factor was found to be associated with an inferior survival in patients with diffuse large B-cell lymphoma (Figure 24) [35]. In time, these technologies will become part of the routine diagnostic services to determine treatment strategies.

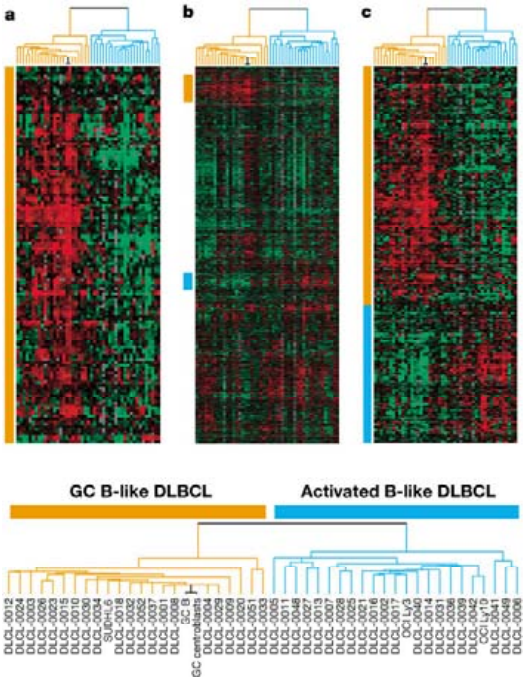


Figure 21. Discovery of DLBCL subtypes by gene expression profiling [33]. Reproduced with kind permission from Pat Brown, Lou Staudt and the publishers of Nature. The samples used in this clustering analysis are shown at the bottom. a: Hierarchical clustering of DLBCL cases (blue and orange) and germinal centre B cells (black). Two DLBCL subgroups, GC-B-like (orange) and activated B-like DLBCL (blue) were defined by this process. b: Discovery of genes that are selectively expressed in GC B-like DLBCL and activated B-like DLBCL. All genes, with the exception of the genes in the proliferation, T-cell and lymph-node gene expression signatures were ordered by hierarchical clustering. Genes selectively expressed in GC B-like DLBCL (orange) and activated B-like DLBCL (blue) are indicated. c: Hierarchical clustering of the genes selectively expressed in GC-like DLBCL and activated B-like DLBCL.

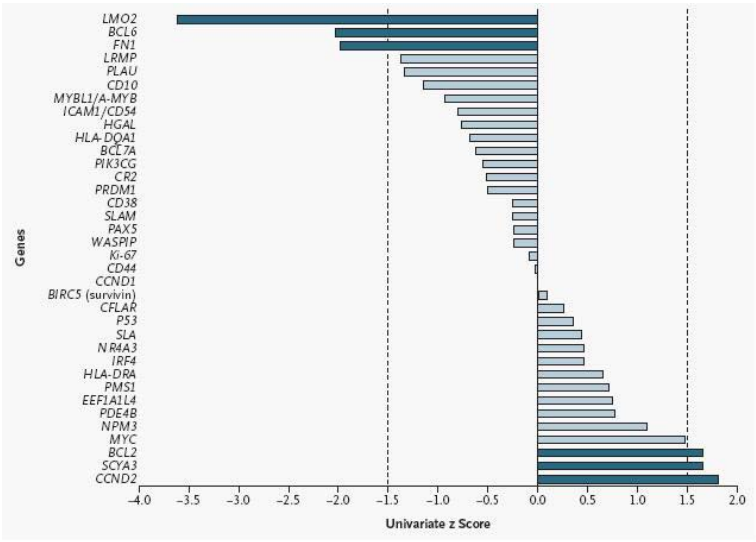


Figure 22.Univariate analysis of expression of 36 genes with overall survival as a dependent variable [34]. Reproduced with kind permission from Ron Levy and the publishers of the New England Journal of Medicine. The genes are ranked on the basis of their predictive power (univariate z score), with a negative score associated with longer overall survival and a positive score associated with shorter overall survival. The dashed lines represent an absolute univariate z score of ±1.5. The prediction model is based on the weighted expression of six genes and is expressed by the following equation: mortality-predictor score
= (-0.0273xLMO2) + (-0.2103xBCL6) + (-0.1878xFN1) + (0.0346xCCND2) + (0.1888xSCYA3) + (0.5527xBCL-2)

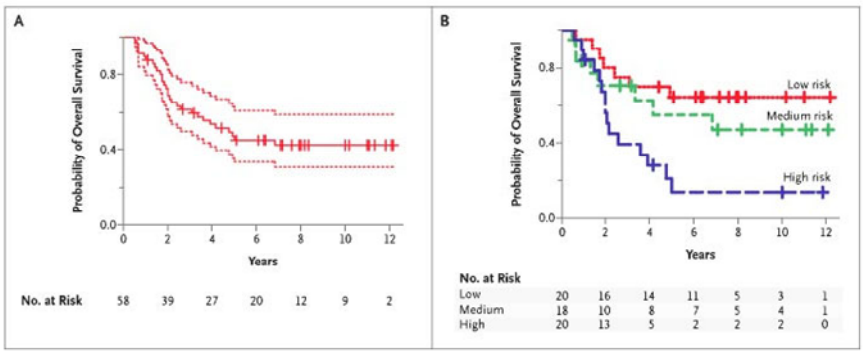


Figure 23. Development of Six-Gene model [34]. Reproduced with kind permission from Ron Levy and the publishers of the New England Journal of Medicine. Panel A shows Kaplan-Meier estimates of overall survival in the 66 patients with diffuse large-B-cell lymphoma, analysed by quantitative reverse-transcriptase polymerase chain reaction with TaqMan probe-based assays. The dotted lines represent 95 percent confidence intervals. Panel B shows Kaplan-Meier curves for overall survival in the three groups (at low, medium, and high risk of death) as defined by a prediction model based on the weighted expression of six genes (LMO2, BCL6, FN1, CCND2, SCYA3 and BCL-2). According to log-likelihood estimates, P=0.001 for the model based on a continuous variable, and P=0.02 for the model based on the three discrete groups shown in the figure.

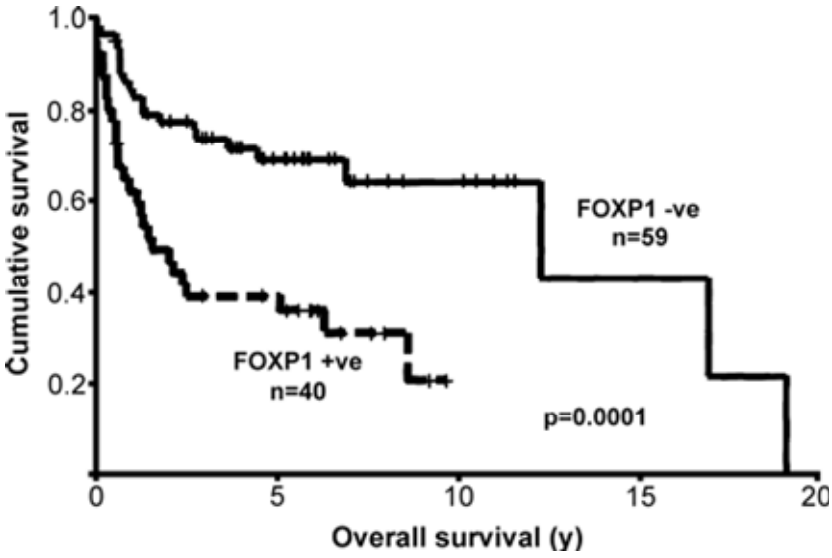


Figure 24. Overall survival for FOXP1-positive versus FOXP-negative expression. Kaplan-Meier overall survival curves stratifying patients according to FOXP1protein expression level [35]. Reproduced with kind permission from Alison Banham and the publishers of Clinical Cancer Research.

6. Concluding remarks

In summary, what is expected of MRD results? Clinicians ask for confirmation of effective therapy, prediction of relapse and type of relapse, time of relapse. The guidelines for therapeutic decision include when to stop therapy or when to modify treatment. It is the hope that combining the PET technology with molecular biology, risk stratification with consequent clinical management and treatment will improve patient care and outcomes. In just over three decades we have identified and cloned the genes responsible for the development of many diseases. The mid 1980s gave us the technology of PCR, which enabled us to screen for gene abnormalities in these diseases. The mapping and sequencing of the human genome is now complete. The translation from the bench to the clinic continues rapidly. These emerging technologies are widely being disseminated in developing countries. In the post genome era we will be able to develop an increasing number of targeted approaches as pathways are being dissected with precision. Together with MRD testing, individualized designer therapy is a reality. The use of radioactivity continues to play a role particularly in imaging and remains the most sensitive method for detecting molecular abnormalities.

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