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Developing and Understanding Biofluid Vibrational Spectroscopy: A Critical Review

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Vibrational spectroscopy can provide rapid, label-free, and objective analysis for the clinical domain. Spectroscopic analysis of biofluids such as blood components (e.g. serum, plasma) and others in the proximity of the diseased tissue or cell (e.g. bile, urine, sputum), offer non-invasive diagnostic/monitoring possibilities for future healthcare that is capable of rapid diagnosis of diseases *via* specific spectral markers or signatures. Biofluids offer an ideal diagnostic medium due to their ease and low cost of collection and daily use in clinical biology. Due to the low risk and invasiveness of their collection they are widely welcomed by patients as a diagnostic medium. This review underscores recent research within the field of biofluid spectroscopy and its use in myriad pathologies such as cancer and infectious diseases. It highlights current progresses, advents, and pitfalls within the field and discusses future spectroscopic clinical potentials for diagnostics. The requirements and issues surrounding clinical translation are also considered.

Keywords: *Biospectroscopy, Biofluids, Infrared Spectroscopy, Raman Spectroscopy, Biomarkers, Cancer, Infectious Diseases, Clinical Implementation, Multivariate Analysis*

Introduction

Biophotonic techniques are widely used in research for developing new modalities with the aim to improve patient healthcare *via* better diagnosis, prognosis, and surveillance. Vibrational spectroscopy holds such promises because the "molecular fingerprint" that it provides represents a snapshot of the sample biomolecular composition and variations therein can be exploited to identify different pathologies [1, 2]. Its sensitivity to such variations makes it possible to probe pathophysiological processes in cells and tissues as demonstrated by many reports for more than a decade [3], leading to the concepts of "spectral cytopathology" and "spectral histopathology" [4-6]. With the advances in spectroscopic/imaging technologies and data processing techniques, cells and tissues can be analysed rapidly and non-invasively to identify disease-related abnormalities. Indeed, some promising studies have reported the added value of vibrational spectroscopy to deliver an objective diagnosis but they were performed on a limited number of patients [7]. In spite of these advances in cell and tissue spectroscopy, the technique has not yet been able to overcome the experimental research phase in order to be transferable from bench to bedside. This is mainly due to the lack of standardisation and validation in large clinical trials and multicentre actions. Access to large sample sets with ethical approval is also a limitation. We believe that spectroscopic diagnosis/prognosis *via* biofluids represents an interesting alternative to cells and tissues. Presently, there is limited research representing high-powered clinical studies for biofluid spectroscopy, yet through the use of animal systems several studies from Naumann's group have set the precedent for studies involving large sample numbers; instilling confidence in the high sensitivity and specificity model outcomes by using several hundred animals per study [8-10].

The quest for disease markers through "liquid biopsies" is a fast emerging field and has only been recently explored by spectroscopic approaches. Blood components like serum and plasma are routinely used for blood testing as they contain biomarkers that are useful for disease diagnostics. For example, in diseases like cancer, they are known to be a rich source of information and represent readouts of the ongoing cellular and extracellular events [11]. Further, they are easily accessible and minimally invasive for patients making large studies feasible. Other organ-specific biofluids in the proximity of the diseased cells or tissues like bile, urine, sputum, and cerebrospinal fluid are also of interest for diagnostic purposes. Recent trends tend to indicate that the use of single or few biomarkers has fallen out in favour of multiple biomarkers [12] and in this context the role of vibrational spectroscopic methods can be determinant as the information provided contains information on global sample biomolecular composition providing a chemical 'fingerprint' or 'signature' of the sample. We will focus on the ability of vibrational spectroscopic analysis to illuminate these disease signatures (disease pattern recognition) for diagnostic purposes as

opposed to the quantitative determination of specific macromolecules within the biofluid [13-15].

This critical review, from both the spectroscopic and clinical points of view, considers the issues encountered during translational research aimed at assessing the potentials of infrared and Raman approaches as rapid and label-free diagnostic methods for biological fluids. In addition, the techniques can be adapted to a variety of diseases and therefore represent a cost-effective investment for healthcare systems. This approach could provide a dynamic diagnostic environment that will enable rapid diagnostics leading to earlier treatment. In addition, the ability to accurately and rapidly monitor disease will allow for closer patient follow-up and earlier change in treatment if needed. This would enable patients to access treatment earlier with reductions in mortality and morbidity.

Vibrational Spectroscopy

Vibrational spectroscopy relates to the specific optical techniques of Infrared (IR) and Raman spectroscopy. These techniques probe intramolecular vibrations and rotations of the sample when irradiated with light [16]. The light-matter relationship is underpinned by the electromagnetic theory postulated by Maxwell [17]. Vibrational spectroscopy has been used for analysing a myriad of samples in chemical, physical and biological applications.

The Raman effect constitutes the spontaneous inelastic light scattering process of photons following the interaction of a monochromatic radiation (e.g., laser source) with the sample. During this interaction both elastic and inelastic scattering processes take place. A high proportion of the photons are elastically scattered with no change in energy (so no molecular information), known as Rayleigh scattering [17]. When photons transfer energy to the molecules as vibrational energy, the energy loss of the scattered photons corresponds to the vibrational energy levels of the molecules. This is known as the Raman-Stokes scattering. The incident photons can in turn receive energy from vibrating molecules, and therefore their frequencies increase, described as the Raman anti-Stokes scattering. Figure 1 shows the transitions involved during these three processes. In spontaneous Raman, the Stokes scattering is generally used due to its higher sensitivity.

Infrared spectroscopy (IR) is broadly defined as the study of absorption characteristics arising from the molecular motion of materials due to atomic displacement [4] upon intimate interaction with an infrared source [18]. Depending on the modality of choice, the radiation can be either transmitted, internally reflected, reflected, or transflected (a combination of transmission and reflectance). During the light - matter interaction, infrared light causes a molecule to enter in a higher vibrational state due to the transfer of ‘quanta’ or ‘packets’ of energy at certain wavelengths dependent upon the

composition of the matter under analysis. Figure 1 illustrates the energy level transition involved in the IR absorption process compared to Raman scattering showing that vibrational energy levels can be probed with both techniques using different physical processes. These transitions result in a spectrum constituted of peaks/bands that can be interpreted qualitatively (peak position) and quantitatively (peak intensity/area, relative intensity). For IR spectroscopy the bands arise from a change in the electric dipole moment of the molecules, whereas Raman is associated with a change in the molecular polarisability.

Constituent chemical molecular bonds present many forms of vibrations which occur at different energies corresponding to different allowed transitions. IR and Raman spectroscopies are complementary and provide a "fingerprint" or "signature" of the molecules contained within the sample depending on whether their bonds exhibit Raman or IR activities. Certain vibrations that are allowed in Raman may be forbidden in IR and *vice versa*. For a full treatise of fundamental spectroscopy works, the authors direct the reader to two reviews by Barth and Haris on IR spectroscopy [19] and Long on Raman spectroscopy [20].

Biological and Biomedical Vibrational Spectroscopy

There is a continuing effort devoted to the exploration of new technologies that can detect early signs of diseases and therefore significantly reduce mortality and morbidity. This depends on the ability to detect biochemical/morphological changes at an early stage of the disease or before the disease becomes symptomatic. Detection of biomarkers plays an important role in this exploration, and in the case of cancer for example, they cover a broad range of biochemical entities, such as nucleic acids, proteins, carbohydrates, lipids, small metabolites, and cytogenetic and cytokinetic parameters, as well as entire circulating tumour cells found in body fluids. They can be used for risk assessment, diagnosis, prognosis, and for the prediction of treatment efficacy and toxicity and disease recurrence.

Over the last 20 years, there has been an exponential increase in the number of studies dedicated to identification of new cancer (Fig. 2a) and infectious disease (Fig. 2b) biomarkers, mainly because of the tremendous development of high throughput molecular technologies and associated bioinformatics. However, among the huge amount of candidate biomarkers, only a limited number have been validated for use in medical practice. A recent paper states that in DNA and proteomic research, out of 1000 biomarkers discovered less than 100 have been validated for routine clinical practice [21].

Vibrational spectroscopy can contribute in bringing a new way for searching biomarkers, namely "spectral signatures" or "spectral biomarkers", which reflect the total biochemical composition of the studied sample as it has been employed for cell and tissue analysis since the pioneering work by Mantsch, Naumann and Diem, to list just a few.

Biological samples are frequently analysed *via* the transmission mode in the mid-IR region, where most molecules absorb and the molecular absorbance is proportional to concentration, obeying Beer-Lambert's law for non-scattering samples. Mid-IR absorption features between approximately 4000 and 400 cm^{-1} (2.5 to 25 µm). Figure 3 illustrates an example of an FTIR spectrum of a breast tissue with the assignment of some important biomolecules. The spectrum can be divided into four regions where the main macromolecules absorb: -CH2 and -CH3 groups of fatty acids and proteins (3050–2800 cm^{-1}); C=O stretching vibrations mainly from lipid esters (1800–1700 cm^{-1}); C=O, N-H, C-N modes from Amide I and II protein bands (1700–1500 cm^{-1}); phosphate vibrations from nucleic acids (1225 and 1080 cm^{-1}); and carbohydrate absorptions (1200-900 cm^{-1}). Libraries housing spectra from biological and biochemical samples have been collected over the years.

Over the years, variants of IR spectroscopic technologies have been tested. A recent review highlights the use of IR techniques to probe the functionality of biological and biomimetic systems [22]. Their applications to study biological and biomedical specimens have continuously increased [23, 24]. When used to analyse biofluids, the mid-IR or near-IR spectroscopies would be performed on drying samples to negate the overwhelming water band from obscuring spectra and to increase automation [25].

Another method of obtaining an IR spectrum is when the sample is placed on a highly reflecting surface, typically aluminium/teflon coated substrates or a glass slide with tin oxide-based silver reflective coating called low e-slides (e.g., MirrIR). In this case the process is termed transflection because the IR beam passes through the sample, is reflected off the slide and passes again through the sample before detection. These substrates have very low cost but recently they have been shown to cause significant spectral intensity variations, due to an electric free standing wave artefact (EFSW) [26, 27] which could be misinterpreted as composition variations while it is the sample thickness variation that is questionable. The fundamental question when using low e-slides is whether the spectral variations observed due to the EFSW impact on the discriminant spectral differences. In case of thin samples such as air-dried cellular monolayers, recent research by Cao *et al.* has shown that the same classification was obtained when performing transmission and transflection measurements [28].

Attenuated total reflectance (ATR) FTIR spectroscopy is a promising modality for biological sample analysis. The guided IR beam propagates through a high refractive index crystal surface producing an evanescent standing wave that penetrates the

sample by a few microns. However for proper use, several issues need to be considered, such as contact between the ATR crystal and the sample, the beam penetration depth and image distortion due to high refractive indices [25, 29].

Despite its molecular specificity, FTIR spectroscopy suffers from some shortcomings which limit its application to the measurement of biological samples and their dynamic behaviour. An important one is sensitivity, in particular in thin samples as a result of the Beer-Lambert's law. Signal amplification can be achieved by the plasmonic resonances of nano-scale metallic particles [30], resulting in the phenomena of surface-enhanced infrared absorption (SEIRA) [31], in analogy with surface-enhanced Raman scattering (SERS) [32]. Early SEIRA studies utilised metal island films [22, 31] and dried samples, but today plasmonic chip-based technology enables the *in situ* monitoring of protein and nanoparticle interactions in aqueous media, at high sensitivity in real time [33].

One method of choice for cell and tissue analysis has been IR microspectroscopy. The coupling of an FTIR spectrometer with a microscope has helped to perform microanalysis and gain in spatial resolution ~ 15-20 µm with a thermal source and ~ 5-10 µm with a synchrotron source using single element detectors. These systems are limited by low sensitivities and time-consuming experiments (several hours) as they remain a point by point acquisition. In the 1990's, the advent of imaging devices with multi-element detectors combined with aperture less microscopes, high-tech automation and faster computers, have drastically reduced the data acquisition times (few hours) with resolution going down to ~2µm/pixel with liquid nitrogen cooled focal plane array detectors. Many research groups have demonstrated the efficacy of employing this to a clinical setting on biopsy samples; minimising subjectivity and increasing diagnostic accuracy [4]. In spite of these progresses, such instruments remain research machines and are not adapted to be used as benchtop techniques for routine analysis in a clinical setting.

The launching of new IR imaging devices incorporating high-intensity tunable quantum cascade lasers (QCL) could revolutionise the way clinical IR images are acquired [34]. High-throughput IR chemical imaging is now in its early days, and needs to be tested and validated. However, a gain of three orders of magnitude in acquisition time has recently been reported for large samples by Bhargava's group [35]. Combining signal enhancement from SEIRA and fast imaging using a QCL source with small bandwidths, a recent study claimed a ~ 200 fold gain in imaging time [36].

The Raman shift covers the range between 0 and 4000 cm^{-1}. Raman spectroscopy can be used in the confocal mode and with the resonance and surface-enhanced modalities. Applications of Raman microspectroscopy for probing biological systems have been continuously expanding over the years along with IR spectroscopy [37]. Its high spatial resolution (~0.5 µm with green lasers), compatibility with aqueous environment [38, 39], and *in vivo* amenability [40-43] makes it a good candidate for biological and biomedical research. Akin

to FTIR, it also provides high content biomolecular information. Microspectroscopy with immersion measurements can be used to enhance signal to background ratio enabling higher quality data acquisition as demonstrated by Bonnier *et al*. [44].

Due to its advantages, label-free, high spectral specificity, limited water signal, and the fact that most biological molecules are Raman active, Raman has been deployed to *in vitro* cell and tissue studies, but now significant developments of *in vivo* work due to the compatibility with fibres, has enabled Raman endoscopy in a label-free manner and *in vivo* Raman probes/endoscopes have made direct tissue analysis possible [45].

New fields of measurement and implementation possibilities have multiplied due to recent hardware developments, improved sampling methods, and advances in the design of Raman technology alongside developments and advances in multivariate data analysis. It has been possible to uncover subtle disease-related spectral changes and exploit them in classification models. However, an important drawback of Raman spectroscopy is that the effect is inherently weak as a very small proportion of incident photons are scattered (~1 in 10^8) with a corresponding change in frequency [17]. This together with the fact that to date most of the commercial systems use dispersive configurations adds another limitation compared to fast IR imaging systems, and makes Raman imaging of biological specimens a slower process. These limitations can be partly circumvented with other Raman modalities based on Resonant Raman Scattering (RRS) and Surface-Enhanced Raman Scattering (SERS) to enable gains in detection sensitivity [37]. In SERS technology, the use of functionalised metal nanosurfaces has allowed optimising the enhancement to several orders of magnitude depending on the metal substrate. Metal nanoparticle arrays and single nanoparticles have been utilized for high-throughput detection [46]. SERS has been applied in different areas in the chemical and biological fields [47] and its very high sensitivity has allowed single molecule detection [48]. Until recently, SERS was not widely applied to biomedical research because of issues linked to complexity of the biological medium, biocompatibility, reproducibility, and short shelf life. However, using silver and gold colloids as SERS substrates, Bonifacio *et al.* recently showed that repeatable spectra could be obtained from protein-free blood serum and plasma [49].

Furthermore, non-linear Raman spectroscopy has been developed to be applied to biomedical analysis like Stimulated Raman Scattering (SRS) and Coherent Anti-Stokes Raman Scattering (CARS), for rapid image acquisition (one Raman band at a time) with higher sensitivities than spontaneous Raman [50-52]. For non-linear Raman, it is important to know which marker band(s) are useful, in analogy to the application of Discrete Frequency–IR (DF-IR) as enabled by the use of QCL sources.

Other areas of current interest for Raman spectroscopy are exploring the sampling depth and location of spectral information. For instance, seminal research conducted by Stone, Matousek and collaborators, demonstrated the principle of spatially offset Raman spectroscopy (SORS) for subsurface analysis towards *in vivo* breast cancer [53, 54] and deep Raman measurements using liquid tissue phantoms to mimic non-invasive cancer screening

applications *in vivo* [55]. Through-tissue sensitivity was increased *via* SESORS measurements at several millimetres depth, i.e., combining SORS with nano-tagged SERS particles [56-59].
Building on the research described above, the field of biospectroscopy has continuously progressed and expanded to complex biological systems such as biofluids [60] with a major focus on the development of a potential diagnostic/prognostic tool with remarkable scope and future clinical promises.

With the global disease burden set to rise, a more rapid, non-invasive, label-free, non-destructive, automatic and cost effective diagnostic technique like vibrational spectroscopy would revolutionise the clinical environment. Its utility as a biofluid diagnostic tool is heavily reliant on the principle that cellular and tissue dysfunction or irregularities affect the biochemical make-up of biofluids, manifesting as protein, carbohydrate, lipid, and nucleic acid subtle differences [16].
Over the last decade, developments in this field have been ongoing in order to fulfil these objectives and ultimately leading to better diagnostics and time to results to improve patient outcomes, offer more efficient public services, and reduce health costs.

Biomarkers in body fluids

According to the National Institutes of Health definition, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention" [61]. In line with this definition, there is a large range of clinical situations where the biomarkers are of paramount importance for the patient's management: screening of patients at risk of the disease or with the disease at an early stage, differential diagnosis of the disease with other conditions, prognosis of the disease independently of the treatment, prediction of the response to treatment, monitoring of disease evolution (Fig. 4).

Molecular biomarkers may be detectable in tissues and biofluids. Figure 5 illustrates the case for cancer where tissue biomarkers can be used for cytological or pathological assessment of the disease or for molecular or spectral imaging techniques. The tumour is vascularised and markers are shed into the bloodstream. Another health issue is the early detection of biomarkers for the diagnosis of infectious diseases coming either from the host or from the pathogen. From the initial interaction onwards the majority of biomarkers available to measure are derived from the host since pathogen numbers are very low and the host is able to utilize components of both the innate and adaptive host response to drive an appropriate response. In serious infection, when pathogens are able to overcome the early host response to their presence, their numbers increase at an exponential rate resulting in significant mortality rates. In such cases, the relative concentration of microbial biomarkers increase over time whilst biomarkers associated with the ongoing, yet

ineffective, host response are still readily detectable (Fig. 6). Preliminary evidence has been produced which indicates that it is possible to identify the presence of an infectious organism through analysis of host biomarker signatures before patients become symptomatic [62]. Thus, the concept of searching for such signatures in host biofluids pre-symptomatically appears as a promising avenue for exploration in order to enable early therapeutic intervention.

Regarding biofluids, blood and its constituents appear the most convenient for biomarker/biosignature detection given its ease of availability and the possibility to repeat the test as often as necessary to monitor disease progression or response to treatment.

Blood serum houses more than 20 000 different proteins. It perfuses all body organs meaning it contains a large range of proteomes from surrounding tissues and cells, making it the most complex biofluid [63]. The low molecular weight fraction serum component of blood, known as the "peptidome" is information rich for diagnostic purposes [11]. Other biofluids (bile, urine, sputum, pancreatic juice, and ascitic, pleural, cerebrospinal fluids), in direct contact with the diseased tissue, are of great interest as media to detect biomarkers/biosignatures that are secreted or shedded locally. These are expected to be present in higher concentration in these fluids than in the blood. In addition, their identification may be facilitated by a less complex molecular composition of local biofluids compared with blood. Although some biofluids such as urine share with blood samples ease of availability and repeatability, analysis of other biofluids requires an invasive procedure, which limits their repeated use in the clinical setting. An example is cerebrospinal fluid which requires a lumbar puncture for collection.

Whilst biomedical vibrational spectroscopy has been developed initially mainly for cell and tissue analysis, it has been also applied more recently to biofluids for biomarker discovery, generating a number of pilot studies with promising results as presented below. The challenge is now to translate the results of these exploratory studies to the routine clinical practice.

Biofluid Spectroscopy

The search for disease markers in biofluids *via* photonic approaches is a fast emerging field and has only been recently explored by vibrational spectroscopic approaches. Biofluids are easily accessible and minimally invasive for patients making large studies feasible. Like cells and tissues, biofluids exhibit vibrational spectra that have characteristic bands reflecting their biomolecular composition. Figure 7 compares the FTIR spectra of some dried biofluids (serum, plasma, and bile) obtained with a high-throughput module in the transmission mode. IR spectra of serum and plasma

present very close profiles with subtle differences that are difficult to depict visibly. This is explained by the fact that serum is essentially plasma with the clotting factors of blood removed. The assignment of the main bands is provided in Table I.
The bile spectrum differs through a higher lipid and carbohydrate content and by relative intensity changes of the protein amide I/amide II bands [64].
Raman spectroscopy gives complementary information to IR. Besides the main macromolecules like proteins, lipids, and carbohydrates, other modes originating from amino acids for example are active. The assignment of the main bands is indicated in figure 8 showing an example of a typical Raman serum spectrum taken from a dried drop.

Serum and plasma

At present, the majority of the biofluid spectroscopy research has focused on serum and plasma. This is most likely due to the prevalence of these types of samples within current biobank stocks or the fact that ethics are already established to collect these samples and all that is required is an addendum stating a separate use of the material.
Malignant diseases - Currently, in the field of oncology, most investigations are proof-of-principle studies showing the potentials of FTIR/Raman spectroscopy to identify different types of cancer from serum samples with high degrees of accuracy. HT-FTIR spectroscopy in transmission mode was used to discriminate urinary bladder cancer patients from patients with urinary tract infection with linear discriminant analysis (LDA) or random forest (RF) classifiers [65]. Using blood serum, Backhaus *et al.* distinguished between breast cancer and controls with a very high sensitivity and specificity [66]. Chemometrics combining support vector machine (SVM) and leave-one-out cross validation was employed by Zhang *et al.* to separate cirrhotic patients with or without hepatocellular carcinoma [67]. Equally important is the possibility to identify liver fibrosis stages prior to the development of hepatocellular carcinoma, which are crucial for the clinical management. A study by Scaglia *et al.* revealed that patients with extensive fibrosis (F3/F4 stages) could be distinguished from those with no fibrosis (F0 stage) on the basis of their FTIR serum spectra using a combination of discriminant wavenumbers [68]. Studies using ATR-FTIR spectroscopy, coupled with classification machine discriminated ovarian [69] and endometrial cancers [70]. It also allowed differentiating glioblastoma multiforme (GBM) from healthy control and low grade gliomas and GBM *versus* healthy control [71, 72].
Applications of Raman spectroscopy to the study of various biofluids from cancer patients are in continuous progress. Sahu *et al.* analysed serum samples and could differentiate oral cancer patients from controls [73]. More recently, they reported that Raman serum spectroscopy was capable to predict the probability of recurrence in this cancer [74]. Other studies have shown the potential of Raman spectroscopy for differentiating normal subjects from patients with breast [75], colorectal [76], or cervical [77]

cancers. A proof-of-concept study using micro-Raman spectroscopy applied to the sera of 71 cirrhotic patients showed that it could be an alternative method for discriminating cirrhotic patients with and without hepatocellular carcinoma [78]. On the other hand, SERS of serum or plasma has also been shown as a promising tool for the diagnosis of various types of cancer such as nasopharyngeal [79-81], digestive [80, 82-84], and prostate cancers [85].

Non-malignant diseases - Serum and plasma have been also employed to diagnose other diseases using biospectroscopy. For example, Raman serum data allowed to differentiate Alzheimer's disease from other dementia [86] and Carmona *et al.* used plasma Raman spectral data to grade mild, moderate, and severe Alzheimer cases [87]. *Via* FTIR spectroscopy of plasma, Peuchant *et al.* have shown that patients with Alzheimer's disease could be well delineated from normal ageing subjects used as controls [88].

Recent plasma data published by Lacombe *et al.* clearly showed that HT-FTIR spectroscopy could be an interesting alternative technique in neonatal screening of rare diseases such as classic galactosemia. Promising results indicated that healthy/diabetic, healthy/galactosemic, and diabetic/galactosemic patients could be discriminated with good sensitivity and specificity [89].

Few large studies have been reported. An example is the study led by Petrich's group showing the potential of mid-infrared spectroscopy in the triage of patients with acute chest pain [90]. This study included 1429 serum samples from 389 patients reporting to two US hospitals (Massachusetts General and Latter Day Saints, Utah) consisted of 104 suffering from acute myocardial infarction (AMI), 136 from unstable angina pectoris, and 149 from chest pain of other sources. FTIR measurements were performed in the transflection mode. Using a threshold value generated from a robust linear discriminant analysis, they achieved high sensitivity and specificity enabling triage of patients with AMI, those most at need within the Accident and Emergency setting, compared to the other sources of chest pain. They hypothesise on the involvement of carbohydrates as discriminant features, possibly a glycation reaction. Interestingly, their results were comparable to the performance of routine cardiac laboratory markers within the same study population. They conclude on the potential of FTIR to aid the diagnostic procedure as early as within the first 6 hours after the onset of chest pain.

Blood plasma from patients has been investigated with Raman spectroscopy as dried drops to identify a reliable biomarker that can differentiate sepsis patients from those with non-infectious systemic inflammatory response syndrome. Neugebauer *et al.* reported on the high sensitivity and specificity that can be achieved [91]. The possibility

of separating the two groups of patients is crucial because a stratification of at risk patients can be established for a rapid delivery of appropriate treatment.
Finally, following the results obtained in a model of infected cultured cells, SERS appears as a promising approach for malaria parasite detection from whole blood [92].

Other biofluids

Other biofluids non-invasively accessible (urine, saliva, sputum, tears) and invasively accessible (bile, synovial fluid, cerebrospinal fluid, amniotic fluid) have been investigated by vibrational spectroscopy for diagnostic purposes.
Somorjai *et al.* were able to distinguish urine samples from normal renal transplants and rejected allografts, applying IR spectroscopy and a three-stage classification strategy [93]. A Raman spectroscopic analysis combined with PCA and quadratic discriminant analysis (QDA) performed on urine, has allowed identification of spectral biomarkers predictive of complications and kidney failure in the urine of diabetic and hypertensive patients [94]. Finally, in the field of oncology, Del Mistro *et al.* reported that SERS using Au nanoparticle substrates had the potential to detect in urine spectral biomarkers of prostate cancer [95].
Another approach by FTIR spectroscopy associated with LDA on saliva, has reported the correct classification of diabetic patients from healthy control [96]. SERS of saliva showed the ability to predict lung cancer by monitoring the decrease of proteins and nucleic acids with 80%, 78%, and 83% accuracy, sensitivity, and specificity respectively [97]. A preliminary study using SERS on saliva suggested the possibility of a quick detection of AIDS but these results obtained on a small number of patients deserve to be confirmed on a larger population [98].
An exploratory study has shown that FTIR spectroscopy applied to sputum could be a useful approach for the diagnostic of the chronic obstructive pulmonary disease [99].
Investigating the potential of human tears for the diagnosis of ocular diseases, Travo *et al.* have shown the discrimination of patients with keratoconus (degenerative disorder affecting the cornea) from healthy control and also between patients at an early or advanced stage of disease by HT-FTIR and PCA [100]. Additionally, Choi *et al.* report that SERS can be used for diagnosis of adenoviral conjunctivitis from tears [101].
Using HT-FTIR spectroscopy in association with support vector machine (SVM) classification and leave-one-out cross validation (LOOCV), Untereiner *et al.* have shown that bile samples of patients with malignant biliary strictures were differentiated from those with benign biliary diseases [64].
Eysel *et al.* using FTIR spectroscopy and LDA with LOOCV on synovial fluid, were able to differentiate samples from joints affected by rheumatoid arthritis, osteoarthritis, spondyloarthropathies, and meniscal injuries [102]. Also from synovial fluid samples, a Raman spectroscopic study associated to a k-means analysis has shown discrimination between patients with osteoarthritis of low or high severity [103].
Liu *et al.* have investigated the amniotic fluid potential for fetal lung development assessments by IR spectroscopy. The lecithin/sphingomyelin (lung surfactants) and lung

surfactant/albumin ratio measurements by IR spectroscopy were quantitatively and qualitatively correlated to those obtained by thin-layer chromatography and fluorescence depolarization, two clinical methods used to determine fetal lung surfactant maturity in amniotic fluid [104]. Prenatal disorders from amniotic fluids have also been investigated by ATR-FTIR spectroscopy revealing spectral profile changes between amniotic fluids from pregnancies with fetal malformations, preterm delivery and healthy term pregnancies [105]. Griebe *et al.* were able by FTIR spectroscopy to distinguish patients with Alzheimer's disease from healthy controls using cerebrospinal fluid [106].

Translation

With a few exceptions, all the mentioned proof-of-concept studies have been carried out on rather small populations and have shown promises for clinical utility and highlight the potential of vibrational spectroscopy for spectral diagnostics. To our knowledge, two major programmes for large scale clinical trials in remote settings are ongoing using hand-held FTIR modalities. The first campaign led by Wood *et al.* concerns the screening of population in Thailand for malarial diagnosis (http://monash.edu/news/show/infrared-light-puts-malaria-to-the-test).
A similar approach is being taken in the UK with the establishment of Glyconics Ltd. Glyconics is using sputum to diagnose Chronic Obstructive Pulmonary Disorder and are moving towards clinical validation of handheld ATR-FTIR on a subset of the UK population (http://www.glyconics.com/technology.asp).

These steps towards actual clinical environment testing is pushing the field to the forefront of the application and will illuminate the utility of these techniques as well as barriers to clinical implementation that need to be overcome.

Multivariate Analysis

It is becoming more and more evident that vibrational spectroscopy represents an interesting approach to explore the diagnostic potentials of circulating biomarkers/biosignatures in various body fluids [60]. Along with the technological development, the front-end sample preparation challenges and approaches, and the data acquisition procedures, the pre-processing and post-processing of spectral data are equally important for the deployment of various biofluid classes into diagnostics development. Vibrational spectroscopic data are inherently multivariate by nature and their pre- and post-processing require multivariate data analysis approaches.

Different instruments from different manufacturers have different responses and spectral distortions and backgrounds have to be taken into account via pre-processing algorithms in order to compare data from different studies for example. The pre-processing should therefore be able to give accurate, robust and reliable data. These considerations should also include how the sample is prepared and conditioned, the

optical substrate used, and the acquisition mode used in order to post-process reliable data. The way the sample is dried or acquired (e.g. transmission or reflection) will also pre-empt the pre-processing procedures. For example, rapid drying of serum can produce a granulating effect which then causes more scattering/dispersion artefacts and a specific correction has to be implemented. It is clear that the pre-processing is not the same for infrared and Raman spectra of biofluids because the physical phenomena involved are respectively absorption and scattering. In FT-IR spectroscopy the use of an interferometer ensures an excellent intensity and wavenumber calibration. In addition, a background signal is regularly recorded and automatically subtracted to obtain the sample spectrum. For Raman, a day-to-day calibration procedure needs to be implemented to correct for instrument response, and to calibrate the wavenumber and intensity axes. Other experimental considerations include the need to subtract substrate contributions and other physical phenomena such as fluorescence and heating. Biofluid vibrational spectra are therefore corrected, derived (or not), then normalized. As a general rule, it is also important to include prior to the pre-processing steps, a quality test to remove spectra with a poor signal/noise ratio (threshold to be defined depending on the sample nature) and a validated outlier removal routine before post-processing.

The post-processing step includes data mining and the construction of classifiers. Very often, the spectral differences between normal and pathological states are very subtle and the next step is to perform data mining, i.e., a process used to extract the salient information from the spectral data. By using specific algorithms, patterns can be found in large batches of data. Thus, such feature selection procedures can help to identify discriminant spectral features to discriminate between patient groups [67]. However, it is important to note that data mining depends on effective data collection, the size of the datasets, and as well as their pre-processing.

To build classification models, several multivariate approaches have been used and as of today there is no general consensus on which method is the best. In other research fields, numerous linear and non-linear supervised algorithms have been evaluated and a combination of methods like SVM and PLS-DA has been shown to enhance the sensitivity and specificity of the classifiers [107]. Generally, building the classifier should include a calibration phase (training phase), an internal validation phase, and an external validation phase (blind testing phase). One of the important issues encountered is the size of the datasets used as a small dataset that does not accurately describe the patient population can lead to under- or over-fitting and impact the classifier outcome. For a classifier to be robust, it is important to have a large number of class-representative patient samples. In addition, the external validation requires a dataset that has not been used in the two previous steps of calibration and internal validation (based upon patient spectra and not replicate

spectra from the same patient i.e. a spectrum from the same patient should not be in the calibration/internal validation and external validation phases). The leave-n-out cross validation method is often used for these models. It is important to note that all spectra from a given patient must be removed in this process in order to enable a valid outcome. Considering all individual spectra, mean spectra or median spectra as input datasets of the classifier should also be taken into consideration although it has been found that when spectra are highly reproducible and after applying a quality control test plus an appropriate outlier removal, the results are comparable [64].

The workflow in figure 9 illustrates the different steps, for both IR and Raman spectroscopies, starting from sample preparation to data pre- and post-processing and the building of classifiers for diagnostics. The issues dealing with pre-processing and post-processing procedures generally used are described in a more detailed manner in a dedicated review elsewhere in this special issue.

Requirements for Clinical Implementation

Over the last 20 years, the number of studies dedicated to identification of new biomarkers has increased exponentially, mainly because of the tremendous development of high-throughput molecular technologies and associated bioinformatics. However, among the huge amount of candidate biomarkers, only a limited number have been validated for use in medical practice [108]. The origin of this discrepancy has been extensively analyzed in the field of proteomics and genomics. Methodological flaws have been identified in the process of their identification and/or clinical validation and recommendations have been set forth to overcome these inadequacies [109-111]. Studies based on vibrational spectroscopy are subject to the same problems. As for other high throughput technologies, the huge amount of data generated by spectroscopic analysis exposes this analysis to a significant risk of false positive findings. This risk should be minimized by rigorously controlling sample and patient related factors in the exploratory phase and by standardizing the conditions of spectral acquisition, processing and analysis (preanalytic/analytic validity). Subsequently, the findings from pilot studies need to be confirmed in independent large cohort of samples (clinical validity) and finally the benefit of using the biomarker/biosignature in the clinical decision-making setting should be clearly demonstrated as well as its favourable medico-economic profile. Only after this process, a newly discovered biomarker can pretend to reach the routine clinical use [109-113].

Preanalytic/analytic validity

In the preanalytic step, attention should be paid to validate sample-related factors and patient-related factors. Standardization of specimen collection and storage is crucial to reach experimental reproducibility not only in an individual laboratory but

also between different laboratories. In addition, investigators should be aware of the risks of contamination during sample handling. In a recent SERS study [49], EDTA, citrate and Li-Heparin used as anticoagulants for plasma collection have been shown to exhibit confounding peaks. When using filtered plasma (with a 3kDa cut-off), contrary to EDTA and citrate, Li-Heparin was filtered out and no longer interfered with the spectral information. FTIR studies have shown that EDTA and citrate spectral contributions can be circumvented using dialysed plasma. In contrast, no interference in FTIR spectra was observed when directly analysing plasma from Li-Heparin tubes [114]. Due to these limitations, serum is often preferred to plasma in spectroscopic analysis. Factors related to patients are of paramount importance to limit the risk of false positives. Inappropriate selection of case patients and control subjects is a common pitfall in spectroscopic studies as widely reported in other high throughput technologies [108, 115]. When comparison groups are not matched for example for age, sex and physical conditions such as hormonal status or pathologies other than the disease of interest, results may be biased and differences identified between groups may be linked to these confounding factors rather than to the disease of interest [109]. Analytic validity includes the technical aspects of the biomarker assessment. In the field of vibrational spectroscopy, the interaction of light with biological molecules is subject to a certain number of drawbacks which should be overcome to meet the criteria of accuracy, reproducibility and robustness.

The most common protocol for spectral analysis of biofluids is the drying of drop deposits. A shortcoming of this method is the heterogeneous drop deposition characterized by the well-known coffee-ring effect, due to the migration of macromolecules towards the periphery of the drop [116-118]. In order to clarify the dynamics of such deposition, Esmonde-White *et al.* used both imaging and Raman spectroscopy to demonstrate that substrate and fluid concentration have a profound effect on dried drop morphology. They showed that the substrate did not affect the chemical composition within the outer ring of the drop whereas the macromolecular concentration has an impact on the spatial distribution of proteins [119]. Using HT-FTIR, Lovergne *et al.* have recently confirmed the impact of serum dilution on the deposition pattern as illustrated in figure 10 [120]. Without dilution, serum spectra were saturated, due to the acquisition in the transmission sampling mode. The 3-fold dilution was shown to be the most suitable for spectral analysis with a good reproducibility and absorbance intensity. The signal/noise ratio was degraded with higher fold dilutions which precludes the analysis of molecules present at a low concentration in the serum. The heterogeneous deposition of macromolecules in the outer ring should be taken into account when using mode point spectroscopic assessment. It has been reported that this issue can be overcome by averaging spectra taken at different points of the outer ring [121]. Another possibility to avoid the coffee-ring effect is to perform an analysis on a film composed of an array of reduced-

size dry drops each formed from 200 pL of serum [122]. The strict control of experimental parameters of drop deposition appears as a major prerequisite to obtain reproducible results [119]. This may be obtained at best by an automated sampling approach as described by Ollesch *et al.* Using this approach, these authors have reported a higher reproducibility of spectral data compared to a non-automatic sampling [122].

ATR-FTIR spectroscopy has been shown to be an interesting approach for the analysis of biofluids as samples can be directly applied onto the ATR crystal without any dilution. However, currently there is no automated device available so that spectral acquisition is time consuming, about 9 times longer than with automated HT-FTIR spectroscopy [120]. The lack of automation is a limiting factor for the transposition of ATR-FTIR spectroscopy into a high-throughput clinical application [123]. This may also be possible when using a high throughput source such as a QCL during a DF-IR approach. However, for limited patient cases, in a hand-held mode it offers advantages of ease of use and ease of sample preparation with no modification/adulteration of the sample. Identically, Raman spectroscopy is also of great interest for biofluid spectroscopy particularly due to developments in hand held technology and immersion Raman which could enable hand held analysis of "wet" serum, negating the need for a drying step.

The technical standardization of spectral acquisition makes sense if reproducible results can be obtained in different laboratories. This external validation is essential on the way towards clinical validity. The inter-instrument transferability is also a challenge that needs to be faced. Finally, the need for automated instruments underline the necessity of a close collaboration between research scientists, clinical practitioners and industrial partners in order to optimize currently available products according to a specific biomedical purpose [1].
Beside the need of standardized spectral acquisition, there is also a need to validate the design of pilot studies including the chemometric analysis. Proof-of-concept studies raise the question of appropriate selection of case patients and controls as discussed below and also the question of sample size. In contrast with classical statistics, there is no simple method to calculate sample size in biospectroscopic studies. However, Beleites *et al.* have proposed in a recent report to use learning curves to determine the appropriate sample size needed to build good classifiers with specified performances [124]. When the number of patients is too limited to divide the population in one training set and one independent validation set, cross validation methods should be used to avoid the high risk of overfitting [125].

Clinical validity

The next step after the phase of pre-analytic/analytic validation is to confirm the diagnostic performance of the biomarker on an independent population of a large number of patients. This means large multicenter randomized control trials where the sensitivity and the specificity of the putative biomarker may be evaluated against the gold standard diagnostic/screening procedure. These studies, particularly the criteria to include case patients and controls, should be carefully designed to demonstrate whether the biomarker is applicable to its specific purpose which may be screening, differential diagnosis, prognosis, treatment response prediction or monitoring of a disease (Fig. 4).

A common mistake is to validate a marker in the diagnostic setting of a disease and then to extrapolate its performance to the screening context. Candidate biomarkers are tested in pilot studies performed in small numbers of patients with patent disease already diagnosed using golden standard methods. It is crucial to validate the value of these markers in the screening context i.e. for early diagnosis in large populations of patients at risk of the disease. The biomarker sensitivity and specificity in the screening target population are usually much lower than in patients with patent disease. In the context of population screening, high specificity is of paramount importance to avoid false positive results, which means patients will be subject to additional diagnostic procedures, potentially invasive and costly for the society. This underlines the necessity of selecting case patients and control subjects according to the clinical setting where the biomarker is intended to be used [109].

A methodology to avoid patient selection bias in screening studies has been proposed by Pepe *et al*. [109, 111, 126]. In the so-called PRoBE study design, samples are collected prospectively in a cohort of patients before the knowledge of the final diagnosis. Once the outcome data becomes available and the diagnosis established, the sample cohort can be used retrospectively by randomly selecting cases and controls. This methodology is promoted by the research consortium "Early Detection Research Network" from the National Cancer Institute to establish specimen reference sets. It has proved efficient for rapid evaluation of potential biomarkers [110].

Clinical utility

A crucial point in the process of biomarker validation before its adoption in routine clinical practice is to demonstrate its clinical decision-making usefulness at an acceptable cost for the society [109]. This means that the positive and negative predictive values of the biomarker should be evaluated in the “real life” patient population since these indicators are dependent on the prevalence of the disease of interest. The difference between clinical validity and clinical utility is illustrated by the debate about the usefulness of Prostatic Specific Antigen (PSA)-based screening program. It is well established that PSA-based screening programs significantly

increase the detection of prostate cancer at an early stage [127]. However, there is also evidence that PSA-based screening carries a high risk of over-diagnosis leading to overtreatment in a significant number of men with early cancer that will never become symptomatic during their life time [128]. Whether the benefits of early detection of asymptomatic prostate cancer outweigh the harms related to over-diagnosis and overtreatment is highly controversial. There is no consensus regarding the clinical relevance of a PSA-based screening program [129]. This emphasizes that, in addition to its diagnostic performance, the biomarker clinical utility has to be demonstrated before its clinical implementation. The clinical utility refers to the balance of benefits to harms and the medicoeconomic evaluation. For this purpose, a validation study should be performed in a large number of unselected patients with clinical endpoints clearly defined to demonstrate the benefit of using a biomarker including quality of life for the patient and socioeconomic aspects for the society [109].

Conclusion

The difficulty in translating biomedical spectroscopy to the clinic is fundamentally based in the fact that after over more than two decades of research, not enough has been done to fully understand the accuracy of these tests with appropriate considerations applied to control groups and limitations of the clinical environment. In addition there is a need to perform large-scale studies to evaluate the spectroscopic tests' efficacy within the clinic. These approaches would also enable a "diplomatic mission" to enable this technology to be acceptable to the medical community through a "hearts and minds" approach. The particular requirements and picture of a clinical spectrometer or suit of spectrometers including the spectroscopic approach should be implemented for different clinical settings, its instrumental requirements (e.g. detector sensitivity and source throughput), and how accurately it can diagnose disease or perform treatment monitoring.

This review has highlighted the increased diagnostic sensitivity observed from the use of biomedical vibrational spectroscopy to analyse biofluids. However, care should be taken for biofluid spectroscopy not to suffer from the identified pitfalls. As the field of biofluid spectroscopy is further researched, a lot of commitment from different stakeholders (researchers, clinicians, and instrument manufacturers) will be necessary to demonstrate its real potential as a rapid, novel, and robust technology to pinpoint "spectral biomarkers / signatures" that can be useful for diagnostic purposes and to predict clinical outcomes, with the promise that the test can be done periodically at low cost for monitoring care.

The initiatives via current networks like the EPSRC CLIRSPEC (http://clirspec.org/), the Raman4Clinics European COST action (http://www.raman4clinics.eu/raman4clinics-a-european-cost-action/) and the 1st International Society for Clinical Spectroscopy

(CLIRSPEC) are currently gearing research, facilities and communities in the clinical spectroscopy arena to achieve these objectives.

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Table I: Assignment of the major absorption bands of a plasma FT-IR spectrum [89]

Bands (cm^{-1})	Major assignments for plasma contents
3300	ν(N-H) of proteins (amide A band)
3055-3090	ν(=CH) of lipids
2950-2960	$\nu_{as}(CH_3)$ of lipids
2920-2930	$\nu_{as}(CH_2)$ of lipids
2865-2880	$\nu_{s}(CH_3)$ of lipids
2840-2860	$\nu_{s}(CH_2)$ of lipids
1730-1760	ν(C=O) of fatty acids
1660	ν(C=O) of proteins (amide I band)
1550	δ(N-H) of proteins (amide II band)
1400	$\nu(COO^-)$ of animo acids
1240	$\nu_{as}(P=O)$ of nucleic acids
1170-1120	ν(C-O) and ν(C-O-C) of carbohydrates

ν: stretching vibrations, δ: bending vibrations , s: symmetric, as: asymmetric. Taken from Lacombe *et al.*, Analyst, 2015, **140**, 2280.

Fig. 1 Energy diagram showing transitions involved during infrared absorption, Rayleigh, Raman Stokes and anti-Stokes scattering. This Jablonski diagram shows that the same vibrational states of a given molecule can be probed *via* two different routes; one directly measures the absolute frequency (IR absorption) and the other measures the relative frequency or Raman shift (Stokes and anti-Stokes). $h\nu_o$= incident laser energy, $h\nu_{vib}$ = vibrational energy, Δυ = Raman shift, υ_{vib} = vibrational frequencies.

Fig. 2 Number of publications returned in PubMed when inputting the term "cancer biomarker" (a) and “infection biomarkers” (b).

Fig.3 FTIR biological spectrum showing frequent biomolecular band assignments from 3,000-800 cm^{-1}, where ν = stretching vibrations, δ = bending vibrations, s = symmetric vibrations and as = asymmetric vibrations. Illustration taken from transmission spectra on human breast ductal carcinoma, prepared on 1mm thick BaF_2 slides [4].The 3000-2800 cm^{-1} region originates mostly from lipids (CH, CH_2 and CH_3 stretching modes), but protein absorption of the same modes also contribute to these absorption bands.

Fig. 4 Schematic of biomarker use in clinical practice.

Fig. 5: Example of potential tumour-site related biomarkers.

Fig. 6 The relative contribution of host and microbial derived biomarkers to enable diagnosis of infection.

Fig. 7 Comparison between HT-FTIR spectra of different biofluids: serum (red curve), plasma (blue curve), and bile (green curve). Spectra are background corrected and normalised. Note: Serum and bile were collected in dry tubes while for plasma samples lithium heparin tubes were used.

Fig. 8 Typical Raman spectrum of dried serum drop with spectral assignments. Spectrum was measured on a calcium fluoride window with a 785 nm laser excitation with an acquisition time of 2x30 seconds.

Fig. 9 Workflow of biofluid spectroscopy from substrate choice through sample preparation to spectral measurements and data analysis with diagnostic classifiers.

Fig. 10 Analysis of dried serum drops and coffee ring effect with different dilutions: white light images (left) and chemical images constructed on amide I protein band (right).

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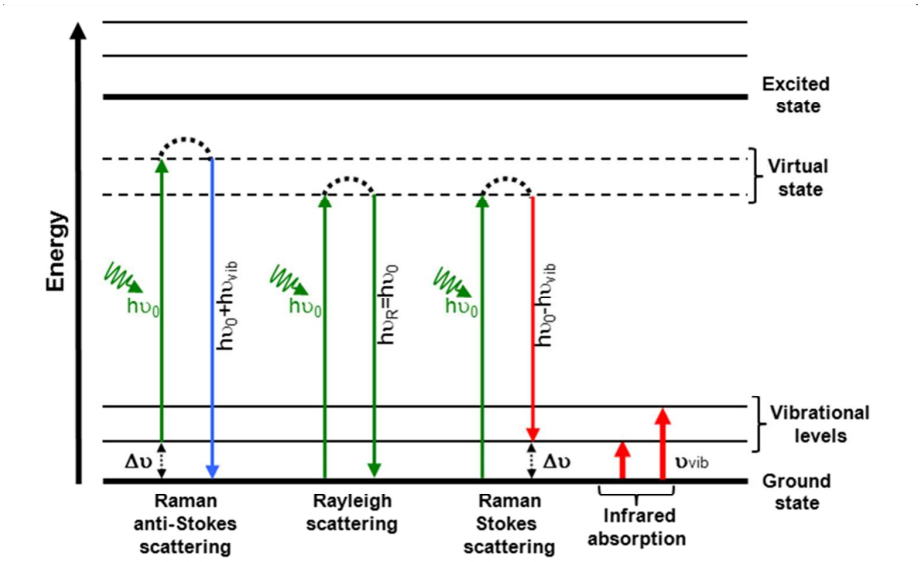
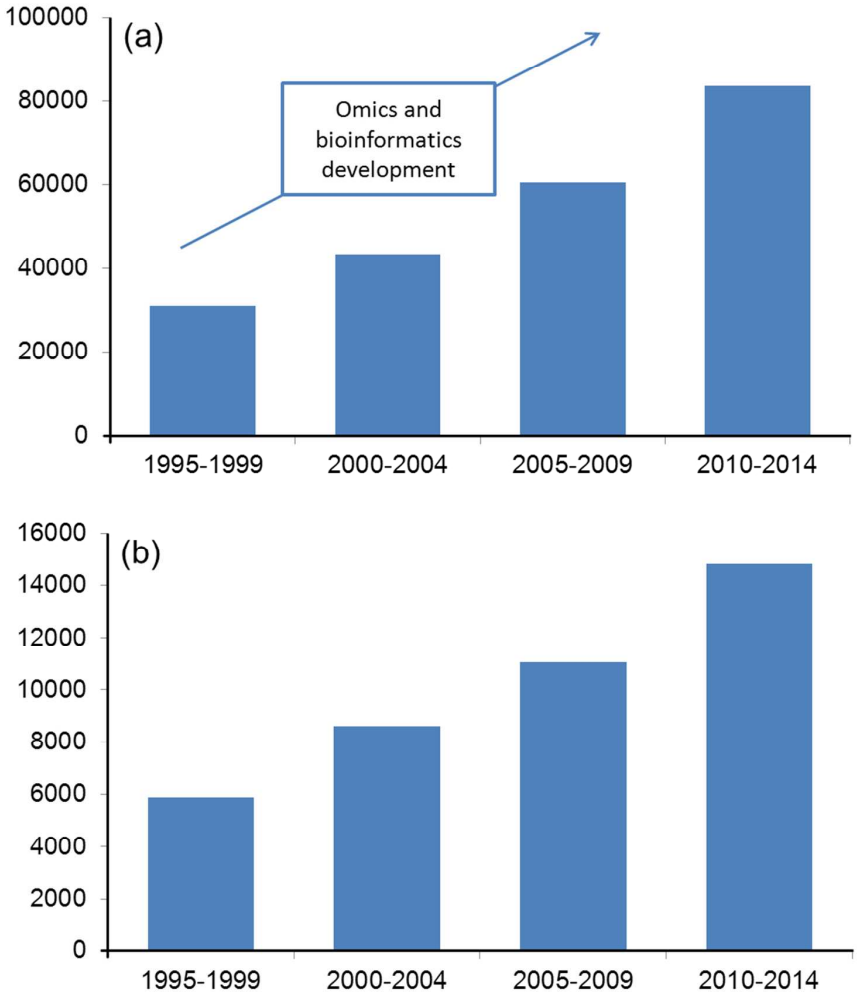


Energy diagram showing transitions involved during infrared absorption, Rayleigh, Raman Stokes and anti-Stokes scattering. This Jablonski diagram shows that the same vibrational states of a given molecule can be probed via two different routes; one directly measures the absolute frequency (IR absorption) and the other measures the relative frequency or Raman shift (Stokes and anti-Stokes). hvo= incident laser energy, hvvib = vibrational energy, Δυ = Raman shift, uvib = vibrational frequencies.
182x117mm (111 x 111 DPI)



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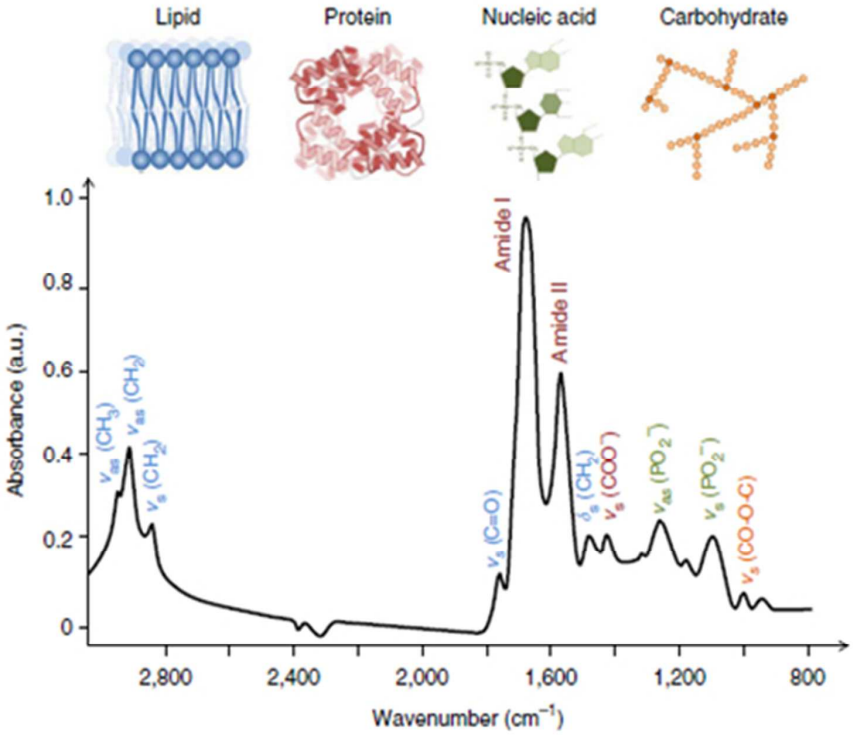
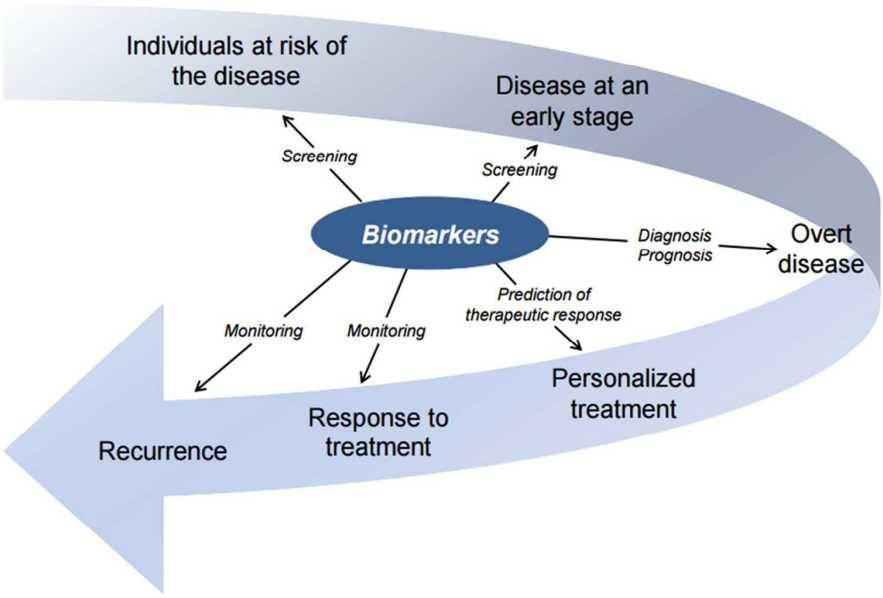
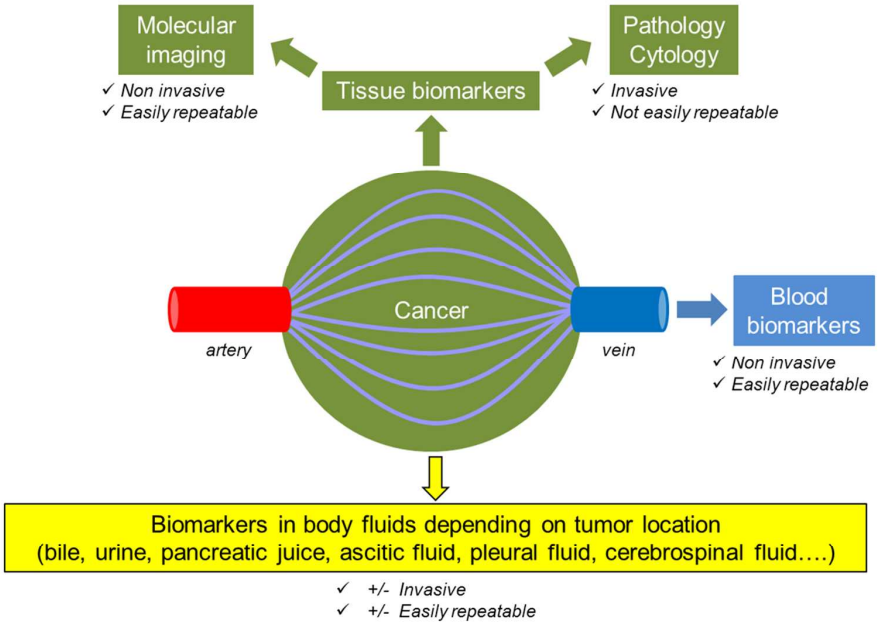


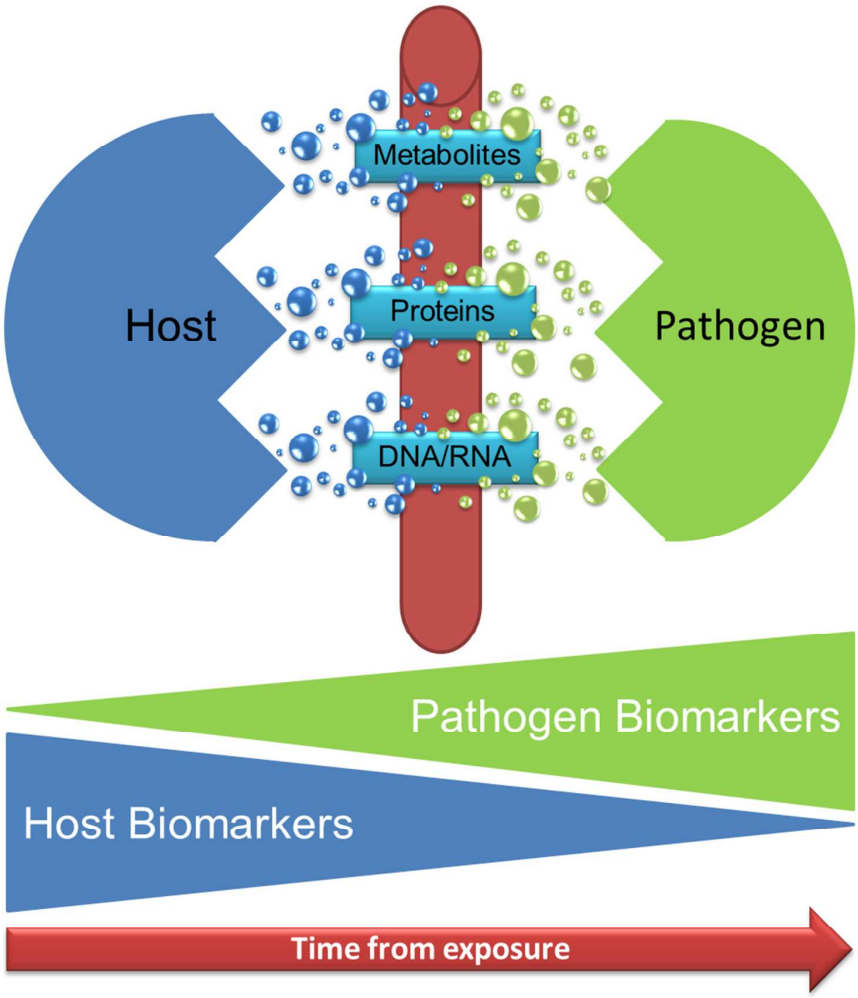
FTIR biological spectrum showing frequent biomolecular band assignments from 3,000-800 cm-1, where ν = stretching vibrations, δ = bending vibrations, s = symmetric vibrations and as = asymmetric vibrations. Illustration taken from transmission spectra on human breast ductal carcinoma, prepared on 1mm thick BaF2 slides.[4]
201x168mm (59 x 61 DPI)



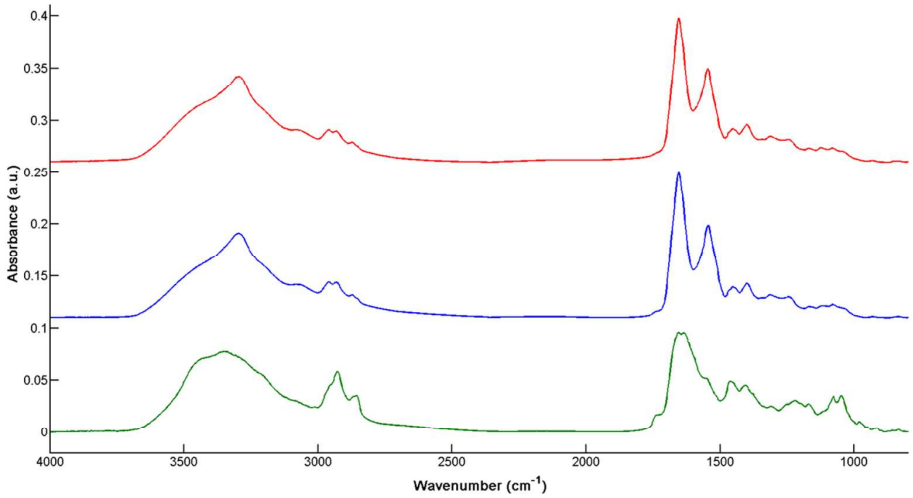
Schematic of biomarker use in clinical practice.
238x168mm (150 x 150 DPI)



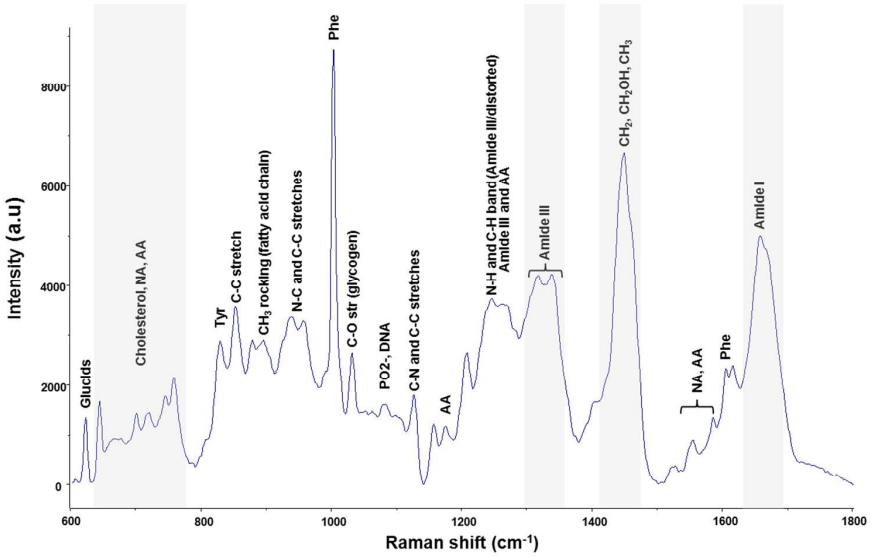
Example of potential tumour-site related biomarkers.
216x154mm (150 x 150 DPI)



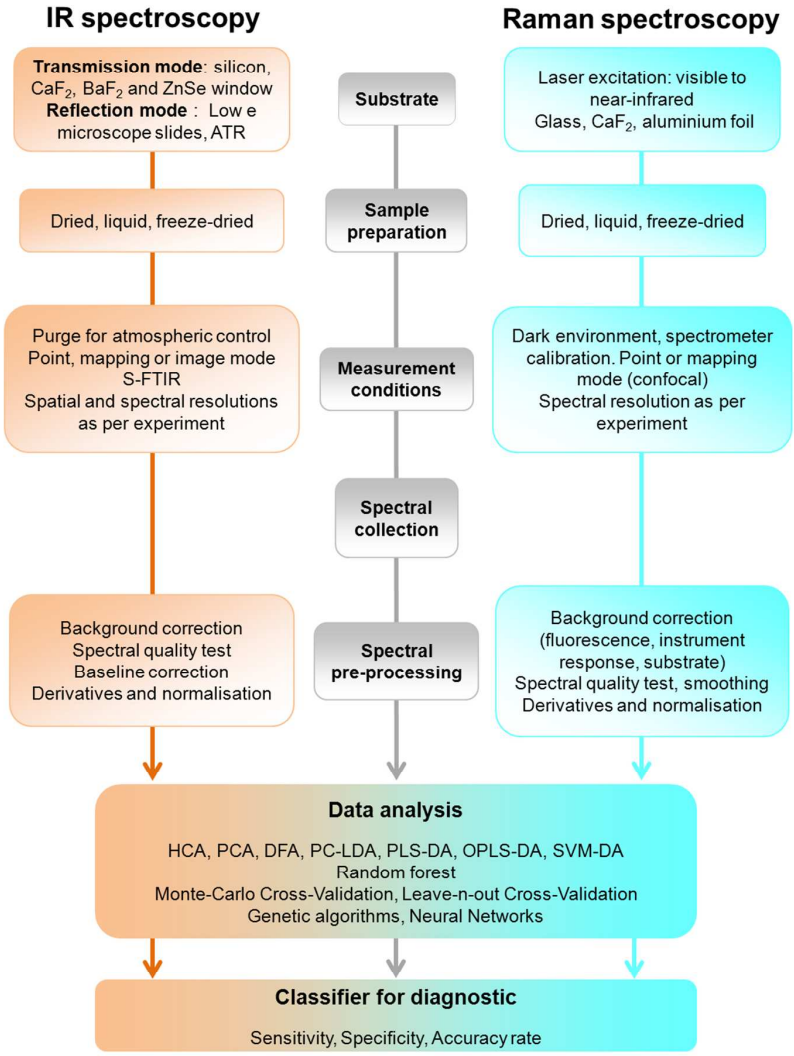
The relative contribution of host and microbial derived biomarkers to enable diagnosis of infection.
172x202mm (150 x 150 DPI)



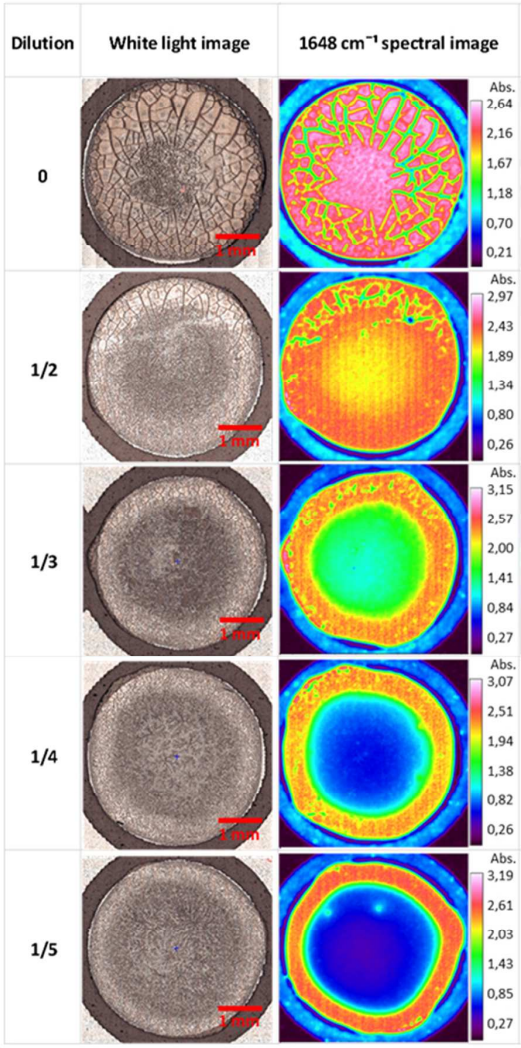
Comparison between HT-FTIR spectra of different biofluids: serum (red curve), plasma (blue curve), and bile (green curve). Spectra are background corrected and normalised. Note: Serum and bile were collected in dry tubes while for plasma samples lithium heparin tubes were used.
209x118mm (150 x 150 DPI)



Typical Raman spectrum of dried serum drop with spectral assignments. Spectrum was measured on a calcium fluoride window with a 785 nm laser excitation with an acquisition time of 2x30 seconds.
218x140mm (150 x 150 DPI)



Workflow of biofluid spectroscopy from substrate choice through sample preparation to spectral measurements and data analysis with diagnostic classifiers.
193x254mm (150 x 150 DPI)



Analysis of dried serum drops and coffee ring effect with different dilutions: white light images (left) and chemical images constructed on amide I protein band (right).
80x154mm (150 x 150 DPI)